ASSESSING INDOOR RESIDUAL SPRAYING FOR MALARIA CONTROL IN CHIKHWAWA, MALAWI, USING EXIT TRAPS ON HOUSES

Thesis submitted in accordance with the

requirements of the University of Liverpool for the

degree of Master in Philosophy

by

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Declaration

The field work described in this thesis was carried out in Chikhwawa, south of Malawi, from October 2010 to April 2012. Insectary assays and Laboratory analysis of mosquito specimen were conducted at Malaria Alert Centre in Blantyre, Malawi and the Liverpool School of Tropical Medicine, UK, respectively.

The study represents original work of my own investigation, and has not been submitted in any degree or diploma to any University. Where work of others has been used, it has duly been acknowledged and bibliography is appended.

Signed.....

(Candidate)

Date.....

ABSTRACT

Rationale

Indoor residual spraying (IRS), using lambda-cyhalothrin, was piloted in Malawi in 2007 by the Presidents Malaria Initiative (PMI) in Nkhota-kota district. The Ministry of Health scaled up IRS to six additional districts across Malawi including Chikhwawa, in 2011. This study was designed to assess the impact of IRS against a background of high malaria prevalence and possible insecticide resistance on the major malaria vectors of Malawi, *Anopheles gambiae* and *An. funestus* in Chikhwawa; and to measure the impact of IRS on entomological indices and malaria prevalence in children of under 5 years of age.

Methodology

Three sentinel sites (Mwingama, Namila and Tsekera) were established in Chikhwawa and 6 window exit traps installed at each site. IRS was conducted in February 2011. Mosquitoes were captured daily, from October 2010 to April 2012, and analysed for species abundance and sporozoites. Separate mosquito collections were carried out using standard WHO insecticide susceptibility assays on *An. gambiae* and *An. funestus* from the sentinel sites. Insecticide quantification of IRS was determined by colorimetric analysis of the wall pads placed on selected houses within the sentinel sites. Anaemia and parasitaemia were determined in children of less than 5 years old from a 50 villages catchment area including the three sentinel sites, through rolling malaria indicator surveys (rMIS).

Results and Conclusion

The study has shown large heterogeneity in mosquito abundance between sentinel sites. Suspected cross resistance found was found in both *An. gambiae* and *An. funestus* to carbamates, organophosphates and pyrethroids suggesting a metabolic based resistance mechanism. Clear resistance (77% mortality) was only found at Namila to deltamethrin in *An. funestus.* There was significant change in resistance pattern at Namila in *An. funestus* to lambda-cyhalothrin between 2011 and 2012 ($X^2 = 6.011$, $p = 0.014$).

No statistically significant change was observed in *An. gambiae* and *An. funestus* abundance differences pre-post IRS suggesting programmatic IRS challenges in Chikhwawa. There was a decline on parasitaemia prevalence from an average of 41% to 29% post IRS.

While entomological surveillance is important for the vector control programme in Malawi, there is a need to utilise this data to improve the actual IRS activities, especially when combined with the results of malaria burden as seen here.

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GLOSSARY

AChE	Acetylcholinesterase
ADD	Agricultural development division
ACT	Artemisinin-based combination therapy
ANC	Antenatal care
ANVR	African network for vector resistance
COMREC	College of medicine research ethics committee
CTPS	Carbamate treated plastic sheet
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHO	District environmental health officer
eMIS	Extended malaria indicator survey
EPI	Expanded programmes on immunization
GABA	Gamma amino-butyric acid
GPIRM	Global plan for insecticide resistance management
GST	Glutathione S-transferase
HCH	Hexachlorocyclohexane
HH	Household
IQK	Insecticide quantification kit
IRAC	Insecticide resistance action committee
IRS	Indoor residual spray
ITN	Insecticide treated net
IVCC	Innovative vector control consortium
Kdr	Knock down resistance
LLIN	Long lasting insecticidal net

LSM Larval source management

MICS Multiple Indicator Cluster Survey

MLW Malawi-Liverpool Wellcome Trust

MFO Mixed function oxidases

MCP Malaria control programme

MoH Ministry of health

NMCP National malaria control programme

OP Organophosphates

PCR Polymerase chain reaction

PDA Personal data assistant

RDT Rapid diagnostic test

rMIS Rolling malaria indicator surveys

WHO World Health Organisation

WHOPES World Health Organisation Pesticides Evaluation Scheme

2. INTRODUCTION

Malaria is the most important of the parasitic diseases of humans with approximately 70% of the population resides in areas infested with potential malaria vectors [1, 2]. The World Health Organisation ranked malaria as the eighth-highest contributor to the global disease burden and the second highest in Africa [3]. Recent estimates of malaria deaths have varied from 0.5 to 3.0 million per year [2, 4, 5] and of 10.6 million yearly deaths in children under 5 years, 8 percent are ascribed to malaria [6]. Malaria control efforts have been intensified in recent years in order to meet Roll Back Malaria, World Health Assembly and Millennium Development targets of universal access and coverage, that aim to prevent, reduce or eliminate disease transmission [7]. The success of these increased control efforts can be seen in the number of countries that have recorded decreases in the number of confirmed cases of malaria and/ or reported reduced admission and deaths since 2000 [2].

In Malawi malaria is endemic and the transmission rates are intense, with seasonal and geographical heterogeneity [8]. *Plasmodium falciparum* malaria is one of the most important public health problems in Malawi, where it is estimated to cause 18.5% of hospital deaths among children <5 years old and one third of all the outpatient visits.

Current strategies to reduce malaria transmission rely heavily on vector control, specifically the use of insecticide treated bed nets (ITNs), indoor residual spraying (IRS), and source reduction [9]. In Malawi, a pilot IRS programme, using lambda-cyhalothrin, was initiated in 2007 in Nkhotakota district, central Malawi, under the President's Malaria Initiative (PMI). This was later scaled up in 2010 by the National Malaria Control Programme to include the six high malaria endemic districts, Chikhwawa, Karonga, Mangochi, Nkhata-bay, Nsanje and Salima districts. In the fight against malaria and the push toward control, elimination and eradication, interventions must be effectively used and accurately evaluated. Insecticide-based malaria vector control is known to result in increasing resistance among the malaria vectors because of the selection pressure placed on the resistance genes [1]. Entomological surveillance to assess the impact of control interventions and monitoring are essential components of any insecticide based malaria vector control programme.

The objectives of this study are;

1. to assess the insecticide resistance status of *Anopheles gambiae* and *Anopheles funestus* the predominant malaria vectors in Chikhwawa
2. to generate a base line of entomological indicators including vector abundance and transmission to monitor by which to measure the impact of vector control

3. to correlate the entomological indices with malaria parasitaemia prevalence before and after vector control

The study was developed within a programmatic setting where the intervention was delivered by the ministry of health (MoH) throughout the district, leaving no option for a contemporaneous control arm. I assumed that there would be a reduction in malaria vector abundance and a corresponding decline in parasitaemia and anaemia prevalence as a result of IRS in Chikhwawa.

The author of this thesis did and supervised all the entomological fieldwork, laboratory preparation of the mosquitoes and insectary bioassays. Mavuto Mukaka, a Malawi-Liverpool Wellcome Trust (MLW) biostatistician, supervised data analysis. Molecular analysis of the mosquito samples to species level, sporozoite detection and wall pad bioassays was done by Miss Kay Hemmings under the supervision of Dr. Mark Paine and Dr. Michael Coleman, from the vector group at Liverpool School of Tropical Medicine. Parasitaemia and anaemia data was provided by Dr. Anja Terlouw, as part of monitoring and evaluation Surveys conducted under the umbrella of the Artemisinin Combination Therapy in Action (ACTia) drug trial within Malawi Liverpool Wellcome Trust in Chikhwawa.

3. LITERATURE REVIEW

3.1. The Global Burden of Malaria

Malaria is a complex and deadly disease that puts approximately 3.3 billion people at risk in 109 countries and territories around the world [2]. Malaria exacts its greatest toll in sub-Saharan Africa countries where approximately 70% of the population resides in areas infested with potential malaria vectors [10]. Approximately 80% of cases and 90% of deaths are estimated occur in the WHO African Region, with children under five years of age and pregnant women most severely affected [2]. Other high risk groups include adolescents [11] non-immune travellers, refugees, displaced persons and labourers from non-endemic areas entering the endemic areas [12]. According to WHO 2012 malaria report, 50 countries are on track to reduce their malaria case incidence rates by 75%, in line with the World Health Assembly and Roll Back Malaria targets for 2015 [2]. However, these 50 countries account for only 3% (or 7 million) of the total estimated malaria cases worldwide. International targets for malaria will be attained if considerable progress is made in the 14 highest burden countries, which account for an estimated 80% of malaria deaths. By 2005, the estimates of global falciparum malaria morbidity burden had increased to 515 million cases, with Africa suffering the vast majority of this toll [13]. Contributing to this resurgence were the increasing problems of *Plasmodium falciparum* resistance to drugs and of the Anopheles vector's resistance to insecticides [14]. The economic burden due to malaria, in Africa alone, is unprecedented, contributing to the cycle of poverty and limiting economic development [15]. For example, Africa alone is estimated to lose at least US$12 billion per year in direct losses (e.g. illness, treatment, premature death), and many times more than that in lost economic growth [1].

Control of malaria is currently on the political agenda of several of the world's wealthiest countries and funds have become available from the Global Fund for Aids, Tuberculosis and Malaria, The US Presidents Malaria Initiative, the World Bank and other bilateral donors to combat malaria, on a scale not seen since the first attempted malaria eradication campaign in the 1950s and 1960 [16]. This substantial increase in funding for malaria control, with effective means for prevention and treatment, is associated with a decline in malaria burden [2].

Some countries have shown impressive gains following expanded vector control and case management. Malaria cases and deaths in health facilities in Rwanda declined by more than 50% between the years 2005 and 2007 in both inpatient and outpatient slide-confirmed cases [17]. Similarly, in Eritrea, between 1998 and 2004, there was substantial reduction in routinely reported clinical malaria cases following scale-up of control measures [18, 19]. Compelling evidence of dramatic decline in malaria transmission has also been reported in Zambia [20-22]; Sao Tome & Principe [23] and Zanzibar (United Republic of Tanzania)[24].

3.2. **Malaria Status, Vectors and Control Interventions in Malawi**

3.2.1. **Malaria in Malawi**

Malaria is endemic throughout Malawi and continues to be a major public health problem, with an estimated six million cases occurring annually. The most prevalent parasite species causing malaria in Malawi

is *P. falciparum* [25]. Two thirds of Malawi's total population, of 14 million persons, are at risk from malaria [26, 27]. This disease accounts for 40% of all hospitalization and 18% of hospital deaths of children less than five years old and 34% of all outpatient visits across all ages [27].

Malaria transmission in many parts of Malawi is seasonal as vector abundance increases with rainfall and temperature. The peak of transmission occurs in the rainy season between November to April especially in low-lying areas with high temperatures. In low-lying areas around Lake Malawi, and the Shire Valley, malaria transmission is intense all year.

3.3. **Malaria Control Interventions in Malawi**

The major malaria control intervention efforts in Malawi include; case-management and intermittent preventative treatment (IPTs).

3.3.1.1. *Chemotherapy*

For many years the treatment of malaria in Malawi relied on chloroquine, sulfadoxine combined with pyrimethamine (SP), and quinine, with the latter being used mainly to treat severe cases. In 1993 Malawi became the first African country to change its first line antimalarial drug from chloroquine to sulfadoxine-pyrimethamine on a nationwide basis in the face of rising rates of resistance to chloroquine [28, 29]. Over the past 5 years artemisinin-based combination therapy (ACTs) have been

introduced. Artemether-lumefantrine (called LA in-country) became the first-line treatment of uncomplicated malaria in 2008 due to resistance concerns in sulfadoxine-pyrimethamine [30, 31]. By 2009, all 42 African malaria endemic countries had changed their policies to support ACT use for uncomplicated malaria [32]. More recently countries have started to adopt policies promoting confirmed malaria diagnosis using malaria microscopy and / or rapid diagnostic tests (RDTs), and Malawi has introduced the use of RDTs in 2012.

3.3.1.2. *Intermittent Presumptive Treatment (IPT)*

Intermittent Presumptive Treatment for malaria in pregnant women (IPTp) is a WHO recommended measure to prevent the consequences of gestational malaria [33, 34]. Intermittent preventive therapy was evaluated for the first time in Malawi in mid 1990's [35]. Clinical trials have confirmed that IPT reduces the incidence of the major complications of gestational malaria, namely anaemia in the mother and low birth weight in the baby and also reduce rates of re-admittance to hospital for severe anaemia or malaria in children [36]. Intermittent presumptive treatment (IPT) involves the administration of a curative dose of an antimalarial drug at predefined intervals to a subject living in an endemic area, without determining whether that subject is parasitic [37]. Malawi IPTp programme has achieved reasonable coverage, but there are increasing concerns about the effectiveness of SP due to the documented spread of drug resistance.

3.3.2. **Insecticide Treated Nets**

While ITNs are often seen as a personal protection measure they can also decrease local malaria transmission by mass killing and decreased survival of *Anopheline* vectors, thereby protecting those in the community without ITNs if coverage and usage is high [38]. Four randomised trials, in Africa, which had child mortality as the primary endpoint, found a reduction in deaths among children under 5 years of +age with the use of ITNs (three trials) or insecticide-treated curtains [39]. A more recent innovation is the long-lasting insecticide-treated net (LLIN), in which insecticide is either incorporated into the fibre during extrusion, or coated on the fibre or the finished net with a binding agent [40].

In Malawi, the current National Malaria Control Programme (NMCP) guidance aims for universal coverage of LLINs which is defined as one net per two people [41]. To achieve this, the NMCP supported a three-pronged approach to LLIN distribution: 1) routine distribution of free LLINs through antenatal care (ANC) and expanded programmes on immunization (EPI) clinics, 2) periodic mass campaigns covering the entire population, and 3) traditional social marketing through private sector outlets. Under the routine distribution channel, the policy states that a pregnant woman should receive a free LLIN either during her first ANC visit or at childbirth if her new-born is delivered in a health facility. In

addition, every child less than one year old receives a free LLIN at his or her first EPI visit [41].

From 2007 pyrethroid-impregnated LLINs have been distributed through antenatal and under-5 clinics at district and central hospitals throughout Malawi [2]. By 2008, approximately 4 million LLINs were procured and approximately 2 million distributed. The percentage coverage for ITN increased between the years 2008 and 2010 from 37% to 42% respectively [2]. In 2012 (after the completion of the data collection for this study) a country-wide distribution campaign was held to support the latest WHO universal coverage targets of 1 net for every 2 individuals.

3.3.2.1. *Indoor Residual Spray*

IRS was included in the Malawi Malaria Strategic Plan of 2011-2015 as a key malaria prevention strategy [41]. It encompasses the application of chemical insecticides onto surfaces where mosquitoes may land and rest indoors in order to kill the adult vector mosquitoes [42]. Scientific evidence of IRS in reducing or interrupting malaria transmission in different epidemiological settings has been available since the 1940s and 1950s [43-45]. Studies have shown that IRS has substantially reduced infant and child mortality [46]. From the year 2000, there has been an increase in the coverage of IRS across the African continent (Figure 3.1). There has been evidence of IRS disrupting malaria transmission, eliminating malaria vectors and reducing malaria incidence [47-49]. South

Africa and Swaziland are good examples of where IRS has successfully controlled the disease to the point that both countries are in currently in elimination phase [50]. More recently in Bioko, Equatorial Guinea IRS has successfully controlled all three major vectors that were responsible for malaria transmission [46, 51].

In the year 2007, the President's Malaria Initiative (PMI) supported a pilot of IRS in Malawi, using Lambda-cyhalothrin, initially covering 27,000 houses in the northern section of Nkhotakota district. This was expanded in 2008 and again in 2009 covering parts of Nkhotakota and Salima districts [52]. However, no indicators were measured for the success or failure of the programme. Following the PMI efforts the NMCP expanded IRS to another five districts across Malawi, covering 500,000 houses and protecting an estimated 2.5 million people [41]. The districts covered included Chikhwawa, Karonga, Mangochi, Nkhata-bay, Nkhotakota, Nsanje and Salima. In 2010, the NMCP used the pyrethroid alpha-cypermethrin, (Morkid) in its five supported districts (including Chikhwawa) while PMI-supported districts changed to an organophosphate, pirimiphos-methyl (Actelic 300cs), due to pyrethroid resistance in the main vector *An. funestus* in that area [53]. Morkid was not on the WHOPES list of recommended compounds for IRS.

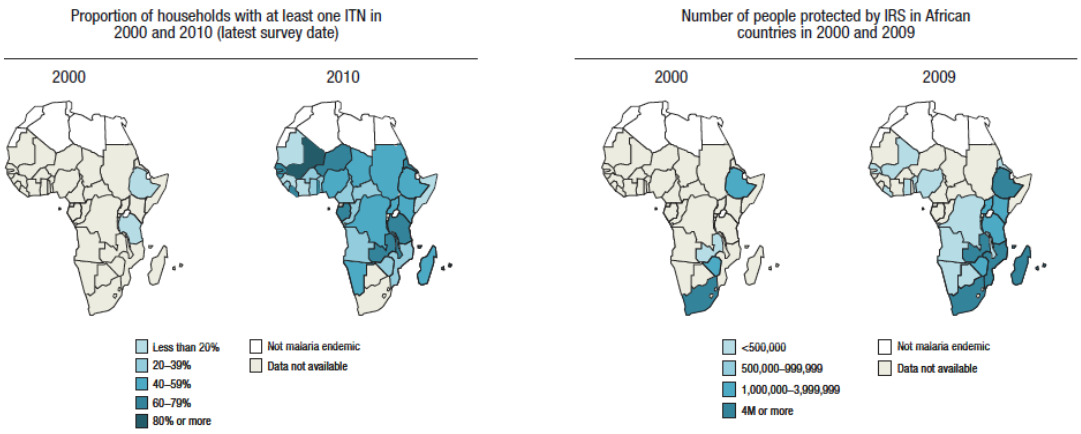

Fig 3.1 Progress in vector control coverage in sub-Saharan Africa from 2000 to 2010, Global Malaria Programme [54].

3.4. **The Malaria Mosquito Vector**

The major malaria vectors in sub-Saharan Africa are *An. gambiae s.s.* Giles, *An. arabiensis* Patton and *An. funestus* Giles (Diptera: Culicidae) [55]. Humans become infected with malaria as a result of their exposure to blood-feeding infectious female *Anopheles* mosquitoes. Blood feeding is critical and obligatory in female mosquitoes as a vital source of proteins essential for egg development and maturation. There are a number of factors that contribute to the unprecedented malaria disease burden in Africa. This region supports the most efficient *Anopheles* mosquito vectors that transmit malaria and are difficult to control as mosquitoes have developed resistance to insecticides commonly used [56]. *Anopheles gambiae* and *An. funestus* are predominant malaria vectors in Malawi with *An. arabiensis* playing a minor role in some areas [25].

3.4.1. **The *Anopheles gambiae* Complex**

The events leading to discovery of the complex *An. gambiae* in the early 1960s have been well described [57]. Research has shown it to be a complex of at least seven morphologically indistinguishable species showing pronounced ecological and behaviour diversity [58-60]**.** These are *An. gambiae sensu stricto* and *An. arabiensis* both present in Malawi [25]. The other species of the complex include; *An. melas* , *An. merus* , *An. bwambae*, *An. coluzzii* and a *An. amharicus* [61, 62]. Three of these sibling species are adapted to fresh-water breeding sites: *An. gambiae* s.s, *An. arabiensis* and *An. quadriannulatus*; two are brackish water breeding: *An. merus* and *An. melas* and one to mineral water: *An. bwambae* [60, 63-65].

Anopheles gambiae s.s. is an efficient vector of malaria and lymphatic filariasis in Africa [66]. Females of this species show a high degree of anthropophily [64] and are therefore a major public health concern. The taxonomy of *An. gambiae s.s* is complicated as this species seems to be in the process of further speciation in West Africa [67]. In West Africa, it exists as two distinct molecular forms, referred to as 'M' and 'S' based on the variation observed in molecular markers [68, 69]. "M form" is named *An. coluzzii* while the "S form" retains the nominotypical name *An. gambiae* Giles [62]. Molecular analysis of the intergenic spacer and the internal transcribed spacer region of rDNA, revealed nucleotide

substitution that differentiated two forms within *An. gambiae s.s.* designated as S and M forms in the case of IGS [68], and Types I and Type II in the case of ITS [70].

Anopheles arabiensis is one of the most recognized malaria vectors in Africa [63, 71]. Even in the presence of other notorious vector species such as *An. gambiae* and *An. funestus*, *An. arabiensis* can play a major role, contributing a large proportion of the infectious mosquito bites that drive intense malaria transmission in communities throughout sub-Saharan Africa [72-75]. This species differs dramatically from *An. gambiae* and *An. funestus* because it is more zoophilic [76] and more outdoor resting and outdoor biting behaviours [63, 74, 77, 78]. *Anopheles arabiensis* frequently feeds on cattle, goats, chickens, dogs, and other available wild and domestic animals. These feeding and resting behaviours complicate the role of *An. arabiensis* in malaria transmission, the ease with which this role is effectively determined through traditional field sampling techniques, and ultimately malaria control [79]. The behaviour of *An. arabiensis* females, makes them only partially vulnerable to IRS [63, 80]. This could result in the maintenance of low malaria transmission even when the *An. gambiae* and *An. funestus* have been controlled using indoor vector control methods. Where, *An. gambiae s.s.* thrives and predominates in humid conditions, *An. arabiensis* is relatively successful in arid zones [81].

Anopheles quadriannulatus is less widespread in its distribution and occurs widely in separated areas of East coast Africa [64, 82]. In Malawi, it has been found widely in southern region [83]. In Zanzibar and Southern Africa, *An. quadriannulatus* was almost completely exophilic while it tends to be endophilic at high altitudes in Ethiopia [64, 84]. This species feeds principally on animals rather than humans [85]. Further studies revealed that the Ethiopian population of *An. quadriannulatus* is a different species and is designated *An. quadriannulatus* B [61]. *Anopheles quadriannulatus* A is found in southern Africa and entirely zoophilic and therefore not regarded as a human malaria vector [61].

Anopheles merus is confined to the east coast of Africa, adjacent inland areas, coastal islands and at inland localities in association with salt pans [63, 64, 86, 87]. This species is predominantly zoophagic [64], but is responsible for low rate of malaria transmission [58] and efficient vector of filariasis transmission in Kenya [88]. *Anopheles merus* plays an unexpectedly important role in malaria transmission in coastal Tanzania [89].

Anopheles melas is a predominant malaria vector in West Africa [63, 64]. This species is known to feed readily on goats and sheep [64]. It was widely considered not to discriminate between man, cow, pig and goat [90]. This vector is partially responsible for transmission of malaria on

Bioko Island, Equatorial Guinea [91] however; it was reported as being controlled by IRS [46].

Anopheles bwambae has only been found from the Semliki forest area of the Uganda/Zaire border, where breeding is apparently confined to hot water springs formed by geothermal activity in the Rift valley [64]. This species is a local vector of malaria and filariasis in the Bwamba County only [65].

3.4.1.1. *Species Identification*

Precise identification of each species has been carried out in isolation using distinct methods, as morphological characterisation for primary identification of members of the *An. gambiae* complex have limited value as it is not feasible to separate out some species this way [63, 64, 92].

Morphologically, there are two characters of salt water species, which are useful in separating them from the fresh water species. Firstly, the eggs of both *An. melas* and An. *merus* are characterised by being longer and broader opening on the dorsal surface than that of fresh water *An. gambiae s.l.* [93]. A much more physical method was introduced for identification of *An. merus* and *An. melas* based on differential response to saline waters, which distinguishes the first instar larvae of the three freshwater-breeding, from those of the saltwater-breeding forms [58]. The method was further extended in the laboratory to include all instars

using colonized *An. merus*, *An. arabiensis* and *An. gambiae s.s.* [94]. Cross-breeding has also been used with much success in elucidating cryptic species. The identification principle is based on hybrid sterility of site-specific hybrids [57]. This technique is not practical for the routine identification of field samples and was largely superseded by genetic [95-97], electrophoretic [85] and polymerase chain reaction (PCR) based techniques [98, 99].

An attempt to investigate the possibility of identifying adults of both sexes of *An. gambiae* and *An. arabiensis* by extracting and analysing their cuticular hydrocarbons was done in the mid-1970s [100]. The preliminary results of this study merit more detailed appraisal of these non-volatile and chemically inert cuticular hydrocarbons for the separation of *An. gambiae* and *An. arabiensis* and other species within the gambiae complex.

Bushrod successfully separated *An. merus* from the fresh water species of the *An. gambiae* complex, *An. gambiae s.s.* and *An. arabiensis,* in Tanzania by plotting the number of coeloconic sensilla against the palpal ratio [101]. Further studies showed that *An. gambiae* and *An. arabiensis* could be distinguished from *An. merus* and *An. quadriannulatus* by the width of the pale band at the apex of hind tarsus three and the base of hind tarsus four [102, 103]. An evaluation of effectiveness of this method

to identifying *An. gambiae* was carried out in KwaZulu Natal, South Africa, and resulted in only 56% correct identification [104].

More recently, molecular methods have been devised which use differences in the DNA polymorphisms to distinguish species by the polymerase chain reaction (PCR) [99, 105-109]. The PCR assay developed by Paskwitz [105] and Scott [99] is based on species-specific fixed differences in the ribosomal DNA (rDNA) region, which includes part of the 28S coding region and part of the intergenic spacer (IGS) . The method uses a universal (UN)21 primer that anneals to a sequence shared by all members of the complex, in combination with specific reverse primers for *An. arabiensis* (AR), *An. gambiae* (GA), *An. quadriannulatus* (QD) and *An. merus* (MR) that bind to unique sequences of each sibling species.

A method involving new primers to identify the two molecular M and S forms within *An. gambiae s.s* was developed by Flavia et al [107]. Fettene developed a PCR to distinguish between species A and B of *An*. *quadriannulatus s.l.* as well other member of the *An*. *gambiae* complex [108]. Fanello *et al*. [109] proposed a new method for differential identification of sibling species in the *An. gambiae* complex, including simultaneous separation of M and S forms within *An*. *gambiae s.s.* This method is a combination of earlier protocols by Scott [99] and Flavia

[107]. To clarify the speciation processes ongoing within, *Anopheles gambiae s.s.* further analysis has been done on the insertion polymorphism of a 200 bp SINE (*SINE200*) within genome areas of high differentiation (i.e. "speciation islands"). This resulted in the development of a new easy-to-use PCR for analysis of genetic differentiation between M and S forms [110].

3.4.2. **The *Anopheles funestus* Group**

Anopheles funestus is a major malaria vector in southern Africa [63]. It includes nine species: *An. funestus*, *An. rivulorum*, *An. vaneedeni*, *An. leesoni*, *An. confuses*, *An. fuscivenosus*, *An. brucei*, *An. parensis*, *An. aruni* [111]. Recently "*An. rivulorum*-like" has been added in the group based on molecular sequencing data [112] and in Malawi *An. funestus*-like was recently identified based on combined molecular, cytogenetic and cross-mating experiments [113]. *Anopheles funestus* larvae thrive in grassy edges or shaded area of permanent and semi-permanent water bodies [55, 63]. It is the only member among the complex that is recognised as an important vector of malaria in Africa characterised by high anthropophilic and endophilic behaviour [63]. The other species of the group are mainly zoophilic and play little or no role in malaria transmission. *Anopheles rivulorum* is only a minor vector at a localised site in Tanzania [114]. Because of the different vectorial capacities, biting and resting behaviours and the close morphologic similarity of members of the *An. funestus* group, accurate identification of field-caught material

is critical for vector control programmes [55, 63]. All these species show morphological overlap at the adult stage, although some species can be identified on egg and larval characteristics [55, 86]. *Anopheles confusus* can only be identified morphologically at egg and larval stage among all the species [113]. A separate PCR to distinguish *An. funestus*-like has now been introduced [113, 115].

3.4.2.1. *Species Identification*

Sibling species belonging to the *An. funestus* group may be difficult to differentiate using traditional taxonomic methods [116]. Only four members of this group, namely; *An. brucei*, *An. confusus*, *An. lesson* and *An. rivulorum*, can be identified using egg and larval morphological classifications [63]. PCR-SSCP assay has been developed to discriminate between four members of the *An. funestus* group namely; *An. funestus*, *An. vaneedeni*, *An. lessoni* and *An. rivulorum* [117]. But because the PCR product show no species-specific size differences when loaded on gel for electrophoresis, a robust PCR has been developed to identify *An. funestus* and *An rivulorum* using the second ribosomal DNA internal transcribed spacer [116]**.** Cytogenetic methods have also been used to identify half gravid female adults of two species; *An. parensis* and *An. funestus* [118].

3.4.3. **Distribution of the *An. gambiae* Complex and *An. funestus* Group in Malawi**

The first studies of malaria vectors in Malawi were carried out in 1921 [119] in the Upper Shire River where two major species; *An. funestus* and *An. gambiae s.l* (referred to as *An. costalis*) were identified to be predominant. Between the years 1922 and 1924, Lamborn carried out collections along the southern lake shore district of Mangochi (then Fort Johnson) and the results confirmed earlier findings of predominance of the two *Anopheliese* species [120]. A survey conducted in 1955 on mosquito vector abundance in the south Malawi singled out *An. funestus* as being in abundant throughout the year and *An. gambiae* in the wet season [121].

More recently, Tambala *et al* confirmed the presence of these species and for the first time identified *An. gambiae s.s* and *An. arabiensis* in the south of Malawi [122]. In 2000, Donnelly & Townson carried out detailed studies on the population structure of *An. arabiensis* in Chikhwawa, southern Malawi [123]. In the same year, Spiers first reported the presence of *An. merus* and *An. quadrianulatus* in Chikhwawa [124]. An investigation of lymphatic filariasis in 2003, established a structured distribution and predominance of mosquito species in Malawi in the following descending order; *An. funestus*, followed by *An. arabiensis* and *An. gambiae s.s.* [125].

Plasmodium falciparum sporozoites have been detected in *An. gambiae s.s, An. arabiensis* and *An. funestus* and all these species have been shown to be the vectors of *Wuchereria bancrofti* in Malawi [126]. The absence of more comprehensive malaria transmission data for Malawi remains a gap in our current knowledge that needs filling, particularly in an era when reducing transmission is increasingly recognised as an important component of malaria control and a necessary step toward eventual elimination of the infection. The interaction between malaria vectors and other diseases also requires further investigation.

3.5. **Insecticides: Classification and Modes of Action**

The World Health Organisation (WHO) has classified public health insecticides into four major groups, namely; carbamates, organochlorines, organophosphates, and pyrethroids [127]. Insecticides are classified according to their chemical composition, origin, toxicological action and their mode of penetration.

3.5.1. **Pyrethroids**

Pyrethroids are a major class of neurotoxin insecticides. They are synthetic analogues of the naturally occurring insecticidal esters of chrysanthemic acid (pyrethrins I) and pyrethric acid (pyrethrins II), originally found in the flowers of *Chrysanthemum cinerafolis* [128]. Pyrethroids are a single insecticide class recommended by WHO for large

scale ITNs and IRS to control malaria transmission because of their high efficacy, rapid rate of knockdown, strong mosquito excite-repellence, low mammalian toxicity and cost efficiency [129]. Full-scale commercial production of pyrethrins from *Chrysanthemum* flowers began in the mid 19th century, the chief ingredients in the extract being pyrethrin I and II which are still in use today in household sprays. However, there general use in agriculture was limited by their low stability in air and light, and the cost of production. Subsequent modification of pyrethrins resulted in the commonly used synthetic pyrethroids namely cyfluthrin, cypermethrin, deltamethrin, flumethrin, lambda-cyhalothrin and permethrin [128]. The pyrethrin and pyrethroid insecticides affect both the peripheral and central nervous systems of insects. They initially stimulate nerve cells to produce repetitive discharges and eventually cause paralysis, an effect similar to, but more pronounced than that of DDT [128]. Pyrethroids also have an irritant effect, causing an excitorepellency response, resulting in hyperactivity, rapid knock-down, feeding inhibition, shorter landing times and undirected flight, all of which reduce the ability of vectors to bite. The target site of this group of insecticide is Na+ channel proteins [130]. Pyrethroids have shorter residual effect of between 4 to 6 months. This necessitates the need to spraying pyrethroids several times a year. Pyrethroids when used for control interventions may require two to four spray cycles per year, depending on the length of the transmission season, with important operational and financial implications for the programmes [131].

3.5.2. **Organophosphates**

Organophosphate (OP) insecticides were discovered in 1854 but their insecticidal properties were only recognised in 1937 [132]. The first organophosphorus insecticide to be developed was tetraethyl pyrophosphate, used as biological warfare during the Second World War in Germany [133]. OPs are generally divided into three groups: aliphatic, phenil and heterocyclic derivates. The phenil OPs are generally more stable than the aliphatics and most of the public health OPs belong to the aliphatic group. Malathion and fenitrothion are the common examples. The OPs act on the mosquito vector by inhibiting acetylcholinesterase, preventing breakdown of the neurotransmitter acetylcholine, resulting in neuromuscular overstimulation and death of the vector [134]. However, OPs generally have relatively short residual effect of between 2 to 6 months depending on the substrate and dosage [131]. However, a new formulation of pirimiphos methyl (ACTELLIC 300 CS, Syngenta) has been shown to be a long lasting (9 months) [135] revolutionising the use of this group of compounds for IRS. However, new insecticides or formulations of existing ones come at a cost, Actellic is approximately 12 times more expensive then the equivalent amount of pyrethroids.

3.5.3. **Organochlorines**

Organochlorines are insecticides that contain carbon, hydrogen and chlorine. They are also commonly referred to as chlorinated hydrocarbons, chlorinated insecticides and chlorinated synthetics. Organochlorines generally belong to four groups: diphenyl aliphatics, hexa chlorocyclohexane (HCH), cyclodienes and polychloroterpenes. The mode of action is to disrupt axon depolarization of the sodium channel [136]. Dichlorodiphenyltrichloroethane (DDT) is the best known diphenyl aliphatic used in IRS. It was the insecticide used predominantly in the WHO eradication campaigns of the 1950s [137]. At the Stockholm Convention on Persistent Organic Pollutants in 2001, the use of DDT was banned for all applications except disease control, because of its environmental effects when used in large volumes. In 2006 WHO reasserted the value of DDT when used for IRS [127]. DDT has long been a cost effective insecticide due to low cost and a long residual efficacy on a surface of 6 to 12 months [131].

3.5.4. **Carbamates**

Carbamates are organic compounds derived from carbamic acid. They were originally extracted from the calabar bean, which grows in West Africa and contain physostigmine, a methylcarbamate ester [138]. The first carbamate insecticide, carbaryl, was introduced in the mid 1950s. A carbamate group, carbamate ester, and carbamic acids are functional groups that are inter-related structurally and often are interconverted

chemically. Carbamate insecticides kill insects by reversibly inactivating the enzyme acetylcholinesterase. They are generally not persistent in the environment. The commonly used carbamates in public health include propoxur and bendiocarb. Carbamates have shorter residual effect of between 2 to 4 months and when used for IRS, often requiring several rounds of spray per disease season [131].

3.6. **Insecticide Resistance**

In Africa the first case of insecticide resistance, involving *An. gambiae s.s* was reported in 1967 in Burkina Faso and was attributed to the use of DDT against cotton pests [139]. Today insecticide resistance is a growing concern in many countries which requires immediate attention [140] (Fig 3.2). Insecticide resistance occurs when an insect develops the ability to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection [1] or failure to achieve the expected level of control when used according to the label recommendation for that pest species [141]. The 21st Century has witnessed a pronounced increase in the use of insecticides for malaria control. Several major donors have invested heavily in long lasting ITNs and IRS activities [137, 142-145]. Currently twelve insecticides are approved by the WHO for IRS, but these belong to just four chemical classes (OPs, organochlorides, carbamates and pyrethroid) and only one class, pyrethroids, is recommended for the treatment of ITNs [146]. These same insecticide classes are also widely used to control agricultural

pests in Africa and this has posed an additional selection pressure on mosquitoes when insecticide contaminated ground water permeates their larval habitats. The intensive exposure to insecticides has resulted in the evolution of insecticide resistance in the *Anopheles* mosquito and other disease vectors [140].

Although resistance is being reported to all classes of insecticides, most new reports are for pyrethroids [147]. This is worrisome, as pyrethroids are the only insecticides that the WHO Pesticides Evaluation Scheme (WHOPES) approved for long lasting insecticidal bed nets and are among the cheapest, long-lasting insecticides for IRS; as such widespread mosquito resistance to pyrethroids may hinder malaria control activities. Trials of insecticide-treated nets with alternative insecticide classes such as carbamate and organophosphate have shown good efficacy [148, 149]. However, a growing number of countries are reporting resistance to more than one class of insecticide, which will restrict options for insecticide resistance management [85].

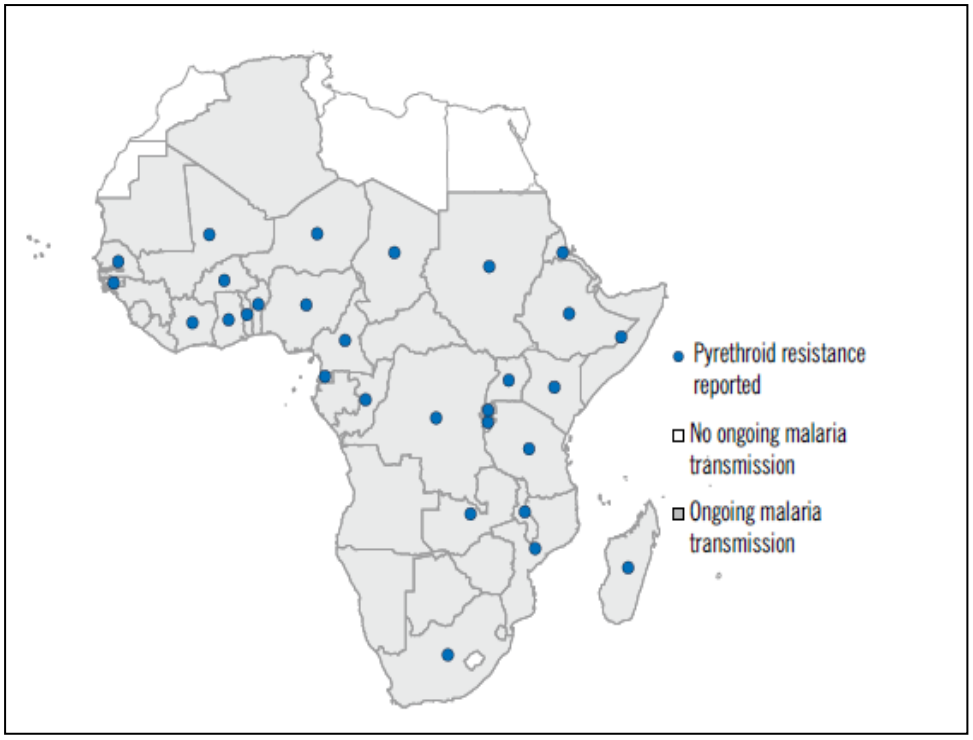

Fig 3.2 Malaria-endemic countries in Africa with reports of resistance to pyrethroids in at least one malaria vector in at least one monitoring site, 2011. (Adopted from WHO Malaria Report, 2011)

In 2012, WHO launched the Global Plan for insecticide Resistance Management (GPIRM), calling for a coordinated response to tackle the growing issue of insecticide resistance [1]. The plan calls on all stakeholders to implement a five-pillar plan:

- Plan and implement insecticide resistance management strategies in malaria-endemic countries;
- Ensure proper, timely entomological and resistance monitoring and effective data management;
- Develop new, innovative vector control tools;

- Fill gaps in knowledge on mechanisms of insecticide resistance and the impact of current insecticide resistance management approaches;
- And ensure that enabling mechanisms (advocacy, human and financial resources) are in place.

Malawi like most effected countries has not yet carried out adequate insecticide resistance monitoring. This means that the global understanding of insecticide resistance is incomplete.

When South Africa, changed from DDT to pyrethroids in the mid 1990s, we were able to observe how insecticide resistance could contribute to the operational failure of a control programme. In brief, *An. funestus* had been eliminated from South Africa due to the use of DDT since the 1950s, however the change to pyrethroids for IRS in 1996 allowed for the pyrethroids resistant population in Mozambique to migrate back into the area. Insecticide resistance of *An. funestus* was reported in 1999 from Kwazulu-Natal province [150]. This resurgence of *An. funestus* was accompanied by an increase in malaria cases and deaths [151]. The increase in disease burden was enhanced due to the escalation of drug resistance at the same time. Lessons were learnt and the malaria control programme (MCP) reintroduced DDT and ACTs bringing malaria under control again.

Subsequently in 2000, *An. funestus* collected from Beluluane, southern Mozambique, were shown to also be pyrethroid resistant and that the

underlying mechanism was an elevated p450 [152]. As the p450 did not give cross resistance to DDT, this explains why South Africa was able to successfully swap back. Subsequent research in southern Mozambique showed that the insecticide resistant population of *An. funestus* extended north of the capital, Maputo [153, 154]. By 2010 *An. funestus* with the same resistance pattern was reported in northern Mozambique [155], which allowed for the assumption that this resistance was moving northwards and had entered Malawi [156]. More extensive research in 2008/09 from 14 sentinel sites across Malawi (Fig 2.2) showed resistance to carbamate and pyrethroids in populations of *An. funestus* [157]. However, there was no evidence of organophosphate or DDT resistance. Further investigation determined that this pyrethroid/ carbamate resistance in *An. funestus* was due to the elevation of two p450's *CYP6P9a* and *CYP6P9b* [157], which is the same as that found in southern Mozambique [158]. At this point in time it is uncertain if this resistance evolved de-novo in several places or spread north through Mozambique and into Malawi.

The impact of insecticide resistance on insect borne disease programs is difficult to quantify [157]. Pyrethroid resistance has been selected in Malawi over the last 3 years in the two major malaria vectors *An. gambiae* and *An. funestus*, with a higher frequency in the latter. The first published data of insecticide resistance in *An. funestus* in Malawi was found from an island in Lake Malawi [156]. Further cases of suspected

pyrethroid resistance in *An. funestus* have been reported in some areas where IRS has been implemented [159]. The impact of this resistance on the ability of either control intervention to reduce disease transmission is poorly understood, and current monitoring and evaluation practices in Malawi are not sufficiently robust to assess this unless lack of efficacy occurs [157].

Elsewhere insecticide resistance monitoring has been used effectively. The Bioko Island Malaria Control Project, high frequencies of the L104F *kdr* allele were observed after an IRS campaign with lambda-cyhalothrin failed to reduce the population density of *An. gambiae*. However due to the successful control of *An. funestus* a modest reduction in transmission index and malaria reported cases was observed [46, 160]. Only after pyrethroids were replaced with the carbamate, bendiocarb did both mosquito populations decline [46].

Another programmatic study was conducted in the highland province of Burundi where a combination of IRS with pyrethroids and ITNs significantly reduced *Anopheles* density by 82% and transmission intensity by 90% despite high frequencies of the L1014S *kdr* allele in the local *An. gambiae* [161-163].

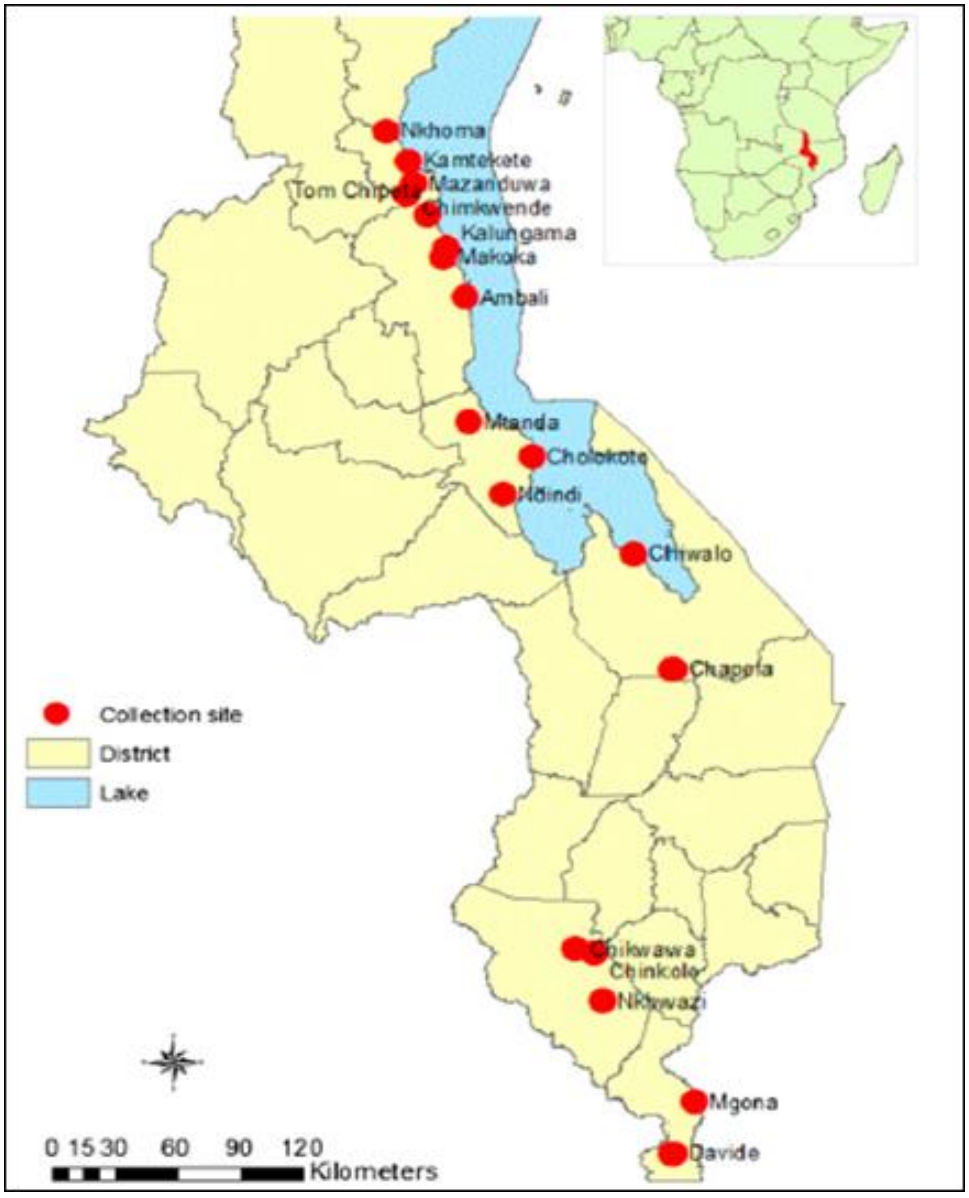

Fig 3.3 Map of Malawi showing localisation of the different collection sites with insecticide resistance reports (from Wondji *et al*, 2012)

3.6.1. **Insecticide Resistance Mechanisms**

Insecticide resistance mechanisms can generally be classified as either;

- metabolic
- altered target site
- behavioural and cuticular

Of these, altered target site and metabolic resistance are considered the two major mechanisms. Altered target site mechanism involves structural changes in the insecticide target sites thereby reducing the insecticide from binding. The metabolic mechanism occurs when increased or modified activities of an enzyme system prevent the insecticide from reaching its intended site of action [147]. More than one resistance mechanism can occur in the same vector population and one mechanism may give resistance to more than one insecticide group. Different types of resistance appear to have different capacities to reduce the effectiveness of insecticide based vector control interventions, with metabolic resistance being considered the stronger and more worrying mechanism [1].

3.6.1.1. *Metabolic Resistance*

Metabolic resistance involves qualitative or quantitative changes in the enzymes, which metabolize or sequester the insecticides before they reach their target sites. It occurs when elevated activities of one or more enzymes results in a sufficient proportion of the insecticide being sequestered or detoxified before it reaches the target site to impair the toxicity of the insecticide [147]. There are generally three enzymes involved in this mode of action namely; esterases, glutathione S-transferase and monooxygenases [164].

3.6.1.1.1. Esterases

Esterase or carboxylesterase is a collective term for the enzymes that hydrolyse carboxylic esters. They comprise of six families of proteins belonging to the α/ß hydrolase fold superfamily [165]. Classification of these enzymes is difficult because of their overlapping substrate specificity [166]. However esterase classification of Aldridge is generally recognised. According to that classification, esterases inhibited by paraoxon in a progressive and temperature-dependent manner are called ß esterases and those which are not inhibited are α esterases [167]. The most common resistance mechanisms in insects are modified levels of esterase detoxification enzymes that metabolize a wide range of insecticides. Increased carboxylesterase activity have been associated with OP resistance in *Culex* mosquitoes, aphids, blowflies and houseflies [168].

3.6.1.1.2. Glutathione S-transferase

Glutathione S-transferases (GSTs) are a major family of detoxification enzymes found in most organisms. All eukaryotes possess multiple GSTs with different substrate specificities to accommodate the wide range of catalytic function of this enzyme family [169]. They catalyse the nucleophilic attack of the endogenous tripeptide glutathione, on a variety of reactive substrates. In early literatures a subset of GSTs are referred to as DDT dehydrochlorinases (DDTases) because of their involvement in dehydrochlorination of DDT to DDE [170]. In mosquitoes, GSTs commonly

confer resistance to DDT [170]. Insect GSTs are now classified into five classes, but previously only two such classes were recognized [169]. In *An. gambiae* seven GSTs have been partially purified which possess 100% of the DDTase activity [170].

3.6.1.1.3. Monooxygenase

Monooxygenases are a complex family of oxidative enzymes involved in the metabolism of xenobiotics. Monooxygenases may also be referred to as cytochrome P450 oxidases or mixed function oxidases (MFOs). They metabolize insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester-oxidation, and nitrogen and thioether oxidation [171]. Cytochrome P450s belong to a vast super family of enzymes. There are 62 families of P450s recognized in animals and plants. At least four families (4, 6, 9, and 18) of cytochrome P450 have been isolated from insects [172, 173]. The *An. gambiae* genome has over 90 P450s genes [173]. The insect's P450s responsible for resistance primarily belong to family six, which, like esterase's, occur in Diptera as a cluster of genes [174]. The cytochrome P450 monooxygenases are involved in many cases of resistance of insects to insecticides primarily to pyrethroids and carbamates, and a lesser extent to organochlorines and organophosphates [175].

3.6.1.2. *Altered Target Site Resistance*

Target-site alteration prevents the insecticide interaction with the target. The most common form of resistance against DDT and pyrethroids, so called knockdown resistance (*kdr*), was first recognised in houseflies in 1951 [176]. Most of these structural changes are as a result of a substitution of a single amino acid in the protein sequence of the target site. There are three major target sites for the four main insecticide families used in public health [177] namely;

- acetylcholinesterase (AChE);
- voltage gated Na+ channel proteins and
- gamma amino butyric acid (GABA) receptor.

In this mode of action, insecticide targets the voltage gated sodium channel on the insects neurone [128]. Insecticide binding delays the closing of the sodium channel prolonging the action potential and causing repetitive neuron firing, paralysis and eventual death of the insect [147].

3.6.1.2.1. Altered Acetylcholinesterase

Acetylcholinesterase (AChE) is the target site for OPs and carbamate insecticides and point mutations in the *Ace* gene are associated with resistance in *Drosophila melanogaster* and *Musca domestica* [178]. Acetylcholinesterase catalyses the hydrolysis of the neurotransmitter, acetylcholine, thereby ending transmission of nerve impulses at synapses of cholinergic neurones in central and peripheral nervous systems [179]. Quantitative and qualitative changes in AChE confer resistance to

insecticides [180]. In resistant insects the enzyme has reduced sensitivity to insecticide inhibition while maintaining its normal function at levels at least adequate for survival. Vaughan *et al.,* demonstrated that the same mutations that cause insecticide resistance in *D. melanogaster* AChE also confer resistance in *Aedes aegypti* [181].

3.6.1.2.2. Altered GABA receptors

The gamma amino butyric acid (GABA) receptor in insects is a gated chloride-ion channel in the central nervous system and in neuromuscular junctions [182, 183]. Altered GABA receptors are the primary target of pyrethroids, avermectins and cyclodiene insecticides [183-185]. All recorded cases of cyclodiene resistance are due to decreased sensitivity of the GABA subtype A receptor [186].

3.6.1.2.3. Altered sodium channel proteins

The Na+ channel proteins in the insect nervous system are the target site for pyrethroids and DDT. Insects with altered Na+ channel proteins are resistant to the rapid knock-down effect of pyrethroids and are called "*kdr*" (knock-down resistance) or "super *kdr*" (highly resistant). These mechanisms have been observed in houseflies (*M. domestica*) [187, 188] and *A. aegypti* [189, 190] and many other insects. In *An. gambiae s.s.*, *kdr* has been reported throughout West Africa [130, 191, 192] and Kenya [193]. It has also been reported in Zambia, the farthest south recorded to date [194]. This resistance is mainly associated with reduced target site

sensitivity arising from a single point mutation in the sodium channel gene, often referred to as knockdown resistance (*kdr*) characterised by a leucine-phenylalanine mutation in West Africa [130].

3.6.1.3. *Cuticular Resistance*

This involves modifications in the insect cuticle and/or digestive tract linings that prevent or slow down the absorption or penetration of insecticides [147]. For malaria control, where insecticides are typically delivered on bed nets or on wall surfaces, the uptake of insecticides is primarily through appendages and therefore insects have evaded this by an increase in the thickness of tarsal cuticle, or a reduction in its permeability to lipophilic insecticides. Microarray experiments have identified two genes cplcg3 and cplcg4, encoding cuticular proteins that are up regulated in pyrethroid resistant strains of *Anopheles* mosquitoes [195, 196]. To date there have been no studies on cuticular resistance in Malawi.

3.6.1.4. *Behavioural Resistance*

Behavioural resistance involves change in insect feeding or resting behaviour (odour repellence) to minimise contact with insecticides in the indoor environment as a result of intensive indoor use of insecticides. Studies in New Guinea and the Solomon Islands showed that the vector *An. farauti* shifted biting times from (23:00 - 03:00) to an early time of 19:00 h after the introduction of indoor DDT before humans were

protected by sleeping in a sprayed room [197]. There is however insufficient data to assess whether these behavioural avoidance traits are genetic or adaptive response [198]. Genetic changes in the mosquito population that shifts feeding or resting behaviour could have a very dramatic impact on the efficacy of the current malaria vector control interventions, potentially exceeding the impact of physiological resistance [147]. To date there have been no studies of behavioural resistance in Malawi.

3.7. **Study Design for Entomological Impact Assessment**

There is limited information on standardised sampling guidelines for entomological impact assessment, unlike those for the human progress, coverage and impact indicators and sampling frames recommended, that are captured in malaria indicator surveys (MIS). Normally, villages or sentinel sites are selected randomly or conveniently from a pool of villages that represent the underlying transmission or intervention. Individual houses are then selected within the cluster, based on specified sampling criteria i.e., intervention coverage, accessibility, urbanity etc. This has formed a routine basis for many monitoring studies, which causes some concern for their representativeness of the underlying population. There is an urgent need for standardised guidelines on sampling frames for entomological surveillance.

3.7.1. **IRS and ITN Monitoring**

Malaria control efforts and elimination in Africa are being challenged by the development of resistance of parasites to antimalarial drugs and vectors to insecticides. ITNs and IRS are currently the preferred methods of vector control especially to suppress transmission in holoendemic and hyperendemic scenarios [199]. Enhanced household level protection can be achieved if the ITNs and IRS have divergent yet complementary properties e.g. highly deterrent IRS compounds coupled with highly toxic ITNs. This works on the principle that if indoor resting mosquitoes are not repelled by insecticide odor from IRS, they must be then killed by the insecticide toxicity on the ITNs. Where both ITNs and IRS are considered, the two methods are mostly used concurrently, within the same household, even though some national strategies emphasize one method more than the other (WHO, 2010). However other than results from a small number of previous trials, which had varied primary objectives [200-202], there has not been any undisputable empirical evidence that ITN-IRS combinations can indeed offer additional communal or personal protection, compared to using either method alone. Studies on whether the combination of ITNs with IRS or carbamate-treated plastic sheeting (CTPS) conferred enhanced protection against malaria and better management of pyrethroid-resistance in vectors than did LLINs alone showed that there was no significant benefit for reducing malaria morbidity, infection, and transmission when combining LLIN+IRS or LLIN+CTPS [203]**.** Similarly, in Eritrea, a study that evaluated the national

malaria control programme between 2000 and 2004, showed that there was no added advantage of using IRS and ITNs as opposed to using either method alone [204]. It was argued that this might have been because the predominant vector in the region, *An. arabiensis* was endophillic and was therefore redundantly affected by ITNs and IRS since these interventions are both indoors. There are also reports showing that even though combination of insecticidal nets with IRS lowered overall vector densities inside houses, there was no overall reduction in malaria transmission relative to situations where one of the methods was used [161, 162]. More recently, Kleinschmidt *et al* completed a review of studies involving both IRS and ITNs in Bioko, Equatorial Guinea and Zambezi province, Mozambique [200] and found that in both places, the odds of contracting malaria were significantly lower for children living in houses with IRS and ITNs, than for children living in houses with IRS alone [200].

Mathematical modelling has also been adopted as a way of estimating potential benefits of combined ITN-IRS interventions [205, 206]. Chitnis *et al*, used a mathematical model to assess the effectiveness of nets and IRS when used singly or in combination in a holoendemic area dominated by *An. gambiae* [205]. It was found that using only ITNs are generally better protected than those with only IRS, and that even though the ITNs or IRS and concluded that a combination of IRS and ITNs would be most effective if the second intervention being introduced is initially targeted

at those people who are not yet covered by the existing intervention [205].

3.8. **Aims and Objectives**

Vector control is critical in reducing malaria transmission to humans and related morbidity and mortality. Continuous entomological surveillance is of paramount importance in insecticide-based malaria control programme to allow for informed decision making on control policy. To implement effective vector based intervention strategies, increased knowledge on interactions of epidemiological and entomological malaria transmission determinants is needed in the assessment of impact interventions. In this regard, shifts in the vector resistance status, species abundance, sporozoite rates and parasite prevalence that have followed in the wake of consistent deployment of these interventions should be monitored to generate pragmatic data for informed policy.

The specific objectives of this study were set to;

1. investigate the species diversity and relative abundance of *An. gambiae* s.l. and *An. funestus* over a one year period in Chikhwawa district, pre and post IRS;
2. monitor insecticide resistance status of *An. gambiae* and *An. funestus* in Chikhwawa district and

3. correlate the entomological indices with malaria parasitaemia prevalence in the three sentinel sites.

3.9. **Study Hypothesis**

The study was set with a hypothesis that IRS implementation reduces the vector population and malaria burden in Chikhwawa. This is based on evidence from similar work conducted in Nkhotakota district which suggested a substantial impact of IRS using lambda-cyhalothrin on parasitemia and anemia prevalence [52]. There are recent reports of pyrethroid resistance in *An. funestus* in Nkhotakota District [157] and Likoma Island, and other parts of Malawi where IRS has been implemented, [156] phenotypically similar to pyrethroid-resistant *An. funestus* in other parts of southern Africa, [150, 152, 154, 158, 207]. This could undermine the efficacy of IRS with pyrethroids and ITNs and therefore close monitoring of the IRS program will be needed to assess the impact of insecticide resistance on IRS efficacy and to guide the choice of insecticide for future spray rounds. Chikhwawa is ideal for this monitoring work because; it is one of the districts covered by IRS, has intense malaria endemicity [41] and a continuous malaria indicator survey has been taking place since May 2010 [208].

4. MATERIALS AND METHODS

4.1. Study design and study period

4.1.1. Overall study design

The core study design is a descriptive longitudinal survey, which allowed a pre- post IRS comparison as part of ongoing daily surveillance of entomological parameters within a group of 18 households from 3 villages, selected to reflect the likely range of malaria transmission within the study area.

The selection of this study design was pragmatic and driven by the available funds and set-up. Due to the programmatic scaling-up of malaria vector control efforts with IRS throughout the entire district, there was no opportunity to implement a more intensive experimental study design to assess IRS intervention efficacy, with a randomized assignment of the intervention of interest. Withholding IRS to villages or households for study purposes was not under our control or ethical at this stage of programmatic roll-out by the. Similarly, an alternative observational cohort study design, whereby households are selected based on the presence or absence of exposure to the intervention of interest was not an option for this study. If the IRS campaign would be successful, all villages and over 80% of households would receive IRS, and those not receiving IRS would unlikely be randomly distributed. Factors that would affect individual households not receiving the intervention would likely include location (hard-to-reach), household refusal, linked to

social economic status /education level or other potential confounders of the association between IRS exposure and impact. Contemporary villages outside the district were not an option either. While the nearest border of the district was relatively close to the selected site, it occurs on the Shire escarpment between Blantyre and the Shire valley in Chikhwawa districts. As the escarpment includes an 800 metre difference in altitude, the transmission setting is entirely different.

Based on this, and due to the limited budget available for my study, I opted for continuous surveillance in a selection of households where the head of the household indicated they would comply with IRS when it would be offered. This would support the 3 objectives, including a pre-post comparison to assess the change in entomological parameters before and after the introduction of IRS. Rolling malaria indicator surveys to determine anaemia and parasitaemia in children of less than 5 years old in the 50 village catchment area including the 3 sentinel sites was carried out in defined periods thoughout the study. A set of households was thus enrolled and monitored over an 18-month period to allow for a comparison of one high-transmission season pre- and post-roll-out. This study design is widely used in programmatic settings, but has a number of inherent study design limitations i.e. lack of contemporaneous control group.

4.1.2. **Study objectives and endpoints**

The aim of the study was to assess mosquito abundance and insecticide resistance status over time, and assess changes pre- and post IRS.

Primary Objective:

1. To investigate the species diversity and relative abundance of *An. gambiae* s.l. and *An. funestus* over a one and half year period in Chikhwawa district, pre- and post IRS.

Primary endpoint:

1. Species specific abundance in the pre-intervention period from October 2010 to February 2011 compared to abundance post-IRS between March 2011 - July 2011 and October 2011-February 2012 periods.
2. Pre- and post IRS comparison of standard transmission indicators (sporozoite rates, transmission indices) between October 2010 and April 2012 periods.

Secondary objectives:

1. Determine level of insecticide resistance to pyrethroids, and other potential alternatives IRS insecticide options.

Endpoint:

WHO standard susceptibility assays.

2. Monitor the household wall level of insecticide over 6 months, following IRS

Endpoint:

Wall Pad colorimetric measurements of the active ingredient of the insecticide used during IRS.

3. Assess consistency of entomological findings with the contemporaneous findings within a continuous Malaria Indicator Surveys within the study area.

Endpoint:

Parasitaemia and anaemia results for children within the catchment area.

4.1.3. **Sample size**

This number of 3 villages and 6 households per village was not based on a sample size exercise, but was adopted from sampling frames that had been previously used in entomological monitoring surveys in Mozambique and Zambia, to ensure consistency [46, 154, 175, 209]. The study was done in these three villages bearing in mind the logistical (all-season road networks) and the prescribed set of criteria as described in section 4.3.

4.1.4. **Study period**

General preparations, installation of exit window traps and the actual mosquito collections for the study started in October 2010. A continuous malaria indicator survey has been carried out in the study area since April 2010. The first IRS was planned for October 2010 but was conducted from February to March 2011 by the MoH spray teams, due to logistical challenges. Figure 3.1 summarises the entire work events for the study.

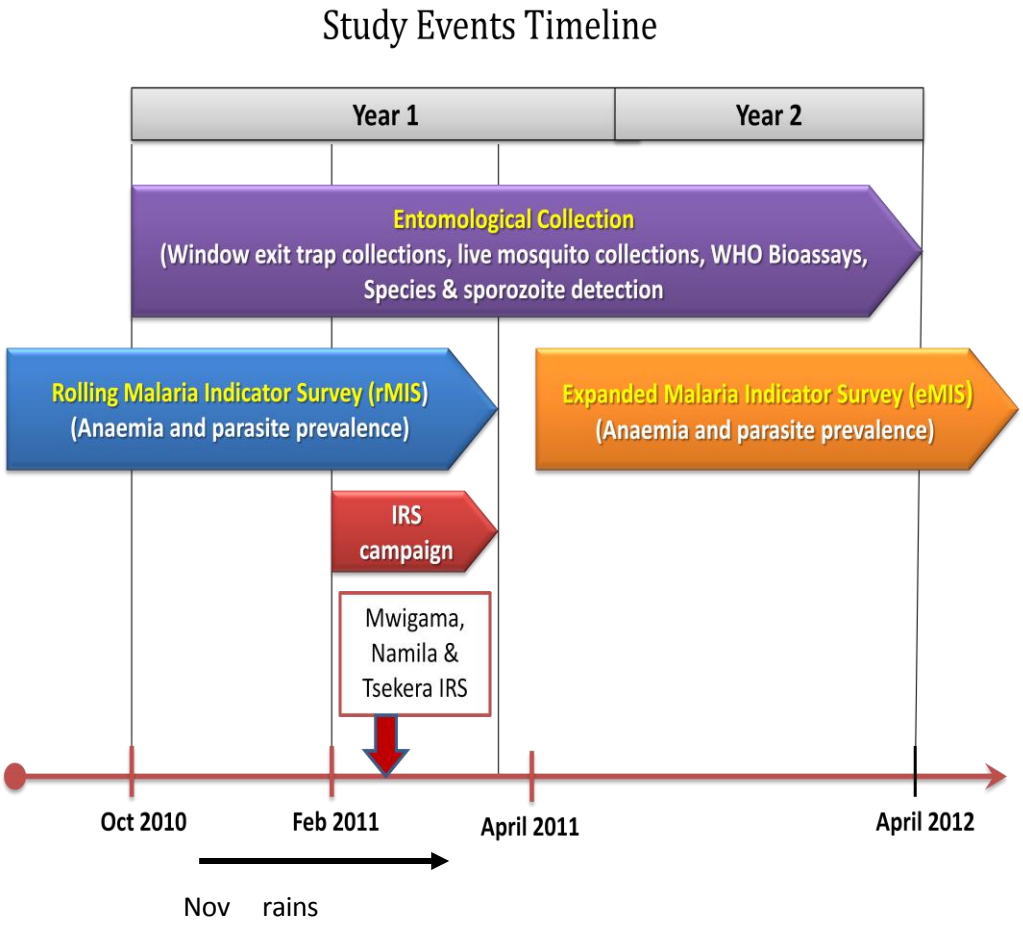

Figure 4.1 Summary of research activities and timeline

4.2. **Study Area and Population**

Malawi, situated in south-central Africa, is a landlocked country bordered by Tanzania to the north, Zambia to the west and Mozambique to the east and south (Figure 4.2). Malawi has an estimated population of 13.1 million, comprised of approximately 17% children less than five years old (Population census, 2008). It has a sub-tropical climate, with three distinct seasons. The warm-wet season stretches from November to April, during which 95% of the annual precipitation takes place. A cool, dry winter season is evident from May to August with mean temperatures varying between 17°C and 27°C, with temperatures falling between 4°C and 10°C. In addition, frost may occur in isolated areas in June and July. A hot, dry season lasts from September to October with average temperatures varying between 25°C and 37°C. Annual average rainfall varies from 725mm to 2,500mm. Humidity ranges from 50% to 87% for the drier months of September/October and wetter months of January and February respectively (http://www.metmalawi.com/climate/climate.php).

Chikhwawa is located in the Lower Shire Valley within the Great African Rift Valley (Fig 4.2 and Fig 4.3) and malaria control has been scaled up considerable over the past 10 years [25, 210-213]. In 2008 Chikhwawa had an estimated total population of 438,895. Annual Population growth rate is 1.1% and an average family size is 4.5 [27].

It is bordered with four districts, namely Mwanza to the North, Blantyre to the North East, Thyolo to the East, Nsanje to the South and Nsanje in the South and it also shares an international border with Mozambique to the West. Chikhwawa has an altitude of about 70m above the sea level and entirely cut through by Shire River, the largest river in Malawi and the only outlet of Lake Malawi, making it viable flood plain for irrigation agriculture. Chikhwawa experiences mean annual temperature of about 26°C with minimum temperatures in June and July and maximum temperatures in October and November. The area receives mean tropical rainfall of 775 mm per annum (district wide data from Nchalo Meteorological Centre).

In terms of agriculture, Chikhwawa has a long history of irrigation, arable and livestock farming (personal communication by the District Agricultural Officer)**.** The total land area is 471, 957 hectares of which 20, 118 hectares is dry arable land and 29,962 hectares is wet arable land (Waterforpeople.org, 2011). It is one of the largest cotton and sugar cane growing districts in Malawi. Cotton is the major cash crop grown in the area and the other crops include maize, millets, sorghum, rice beans and vegetables. Pyrethroids are a major class of insecticides commonly used for growing cotton in the area [214]. From 2005 there has been large scale free distribution and selling of insecticides to farmers at subsidised rates through the Shire Valley Agricultural Development Division (SVADD)

in a programme called the Farmers Input Subsidy (Personal Communication).

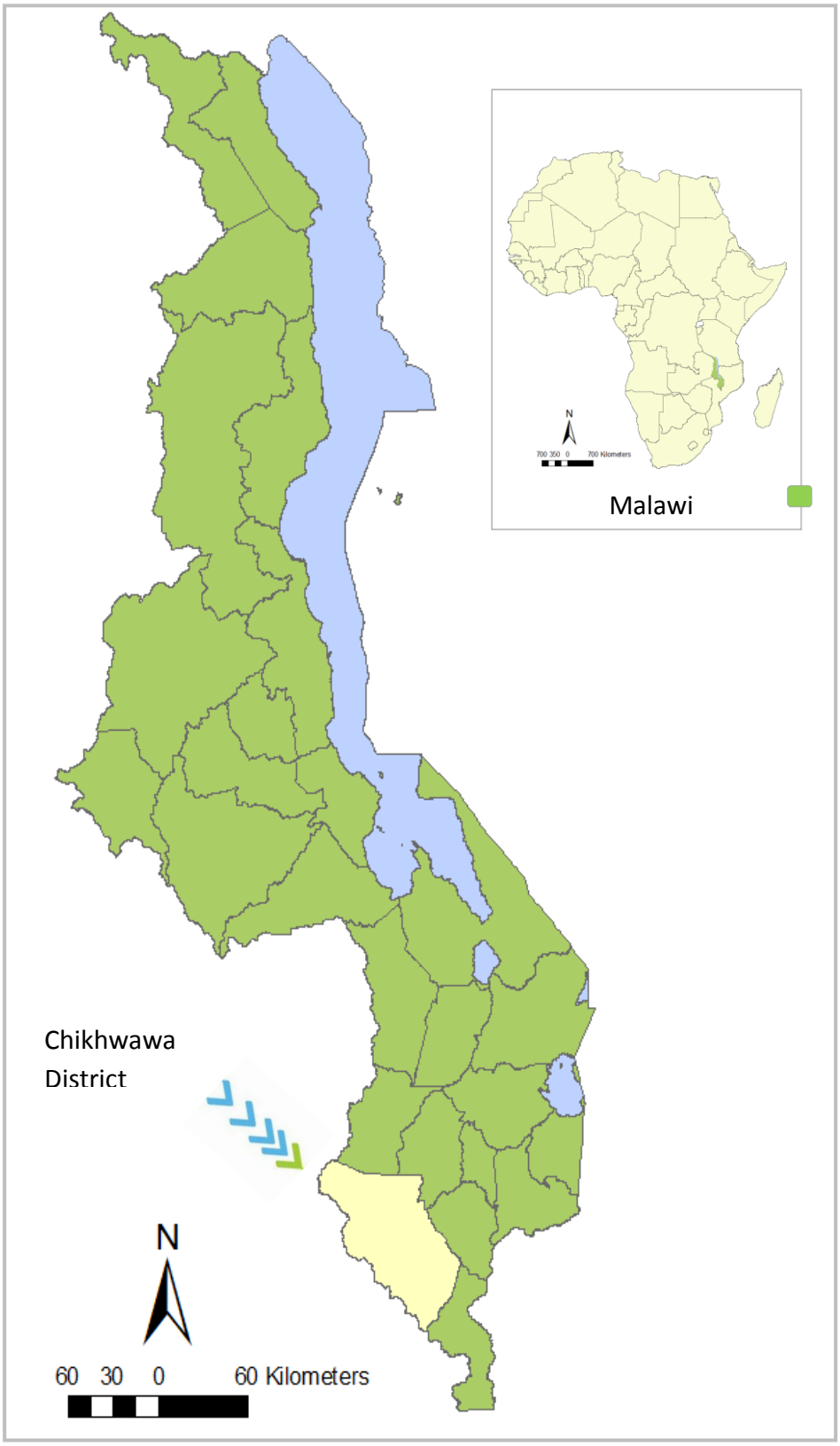

Figure 4.2: Map showing position of Malawi in Africa and the location of Chikhwawa District in Malawi shown in Yellow in the main map.

4.3. **Study Procedures**

4.3.1. **Village and Household Selection**

Following consultation with the District Environmental Health Officer (DEHO), three sentinel sites were selected from the 50 villages in the catchment area of the Artemisinin Combination Therapy in action (ACTia) project [208] in Chikhwawa (16° 1’ S, 34° 47’ E) namely; Mwingama, Namila and Tsekera (Figure 4.2 and 4.3). The 3 villages were selected based on their distance from the Shire River, which provides a source of water for larval breeding and secondly with the aim to capture and represent the baseline variation in mosquito abundance in the area before IRS. One village was selected near the Shire River (<~ 1km), 1 village within 1-9km, and 1 village further from the river (>= 10 km) (Fig 4.3). Eligible villages also had to be accessible easily by road throughout the year to accommodate the monthly mosquito collections and any unannounced spot checks for quality control purposes.

4.3.1.1. *Household eligibility criteria:*

For the selection of households, I prioritized characteristics that would support continuous, accurate collection of mosquito abundance over time, rather than representativeness of households of the overall underlying population. Representative would be difficult to achieve with the small numbers of houses, and the design focused on a pre-post comparison that focuses on capturing change within a single group rather than difference between two groups.

Three standard physical attributes were used to select households for this entomological activity. Firstly, the household needed to be permanent and be available for the whole study period (>1.5yrs). Secondly, the household needed to have a separate kitchen from the bedroom or main house so that kitchen smoke should not darken the white window trap nor confound with mosquito abundance and migration within the house. This aspect has a limitation of increasing the estimates of mosquito abundance. Finally, a consideration was made on choice of houses with minimal open eaves to allow for easy mounting of the exit window trap. This however would operationally reduce the mosquito abundance. The three villages selected for the study had general similarity in terms of the type of houses found; social economic status, agricultural type, literacy levels and general geographical characteristics i.e. soil type and terrain.

With assistance from the village chief or his representative the selected villages were divided into 3 strata of approximately equal size to ensure spatial representation within each of the selected villages. The approximate centre point was determined for each of the 3 strata, and using a list of household eligibility criteria, 2 eligible households were selected nearest to each centre point. If the household owner could not count and read or was less able to independently perform the collection task or declined, the nearest next eligible household was selected, from

the same strata. Prior knowledge of whether the household structure would be permanent or not over the duration of the collection, was also taken into consideration when selecting the households. A total of 6 households were thus selected and window exit traps installed in each village.

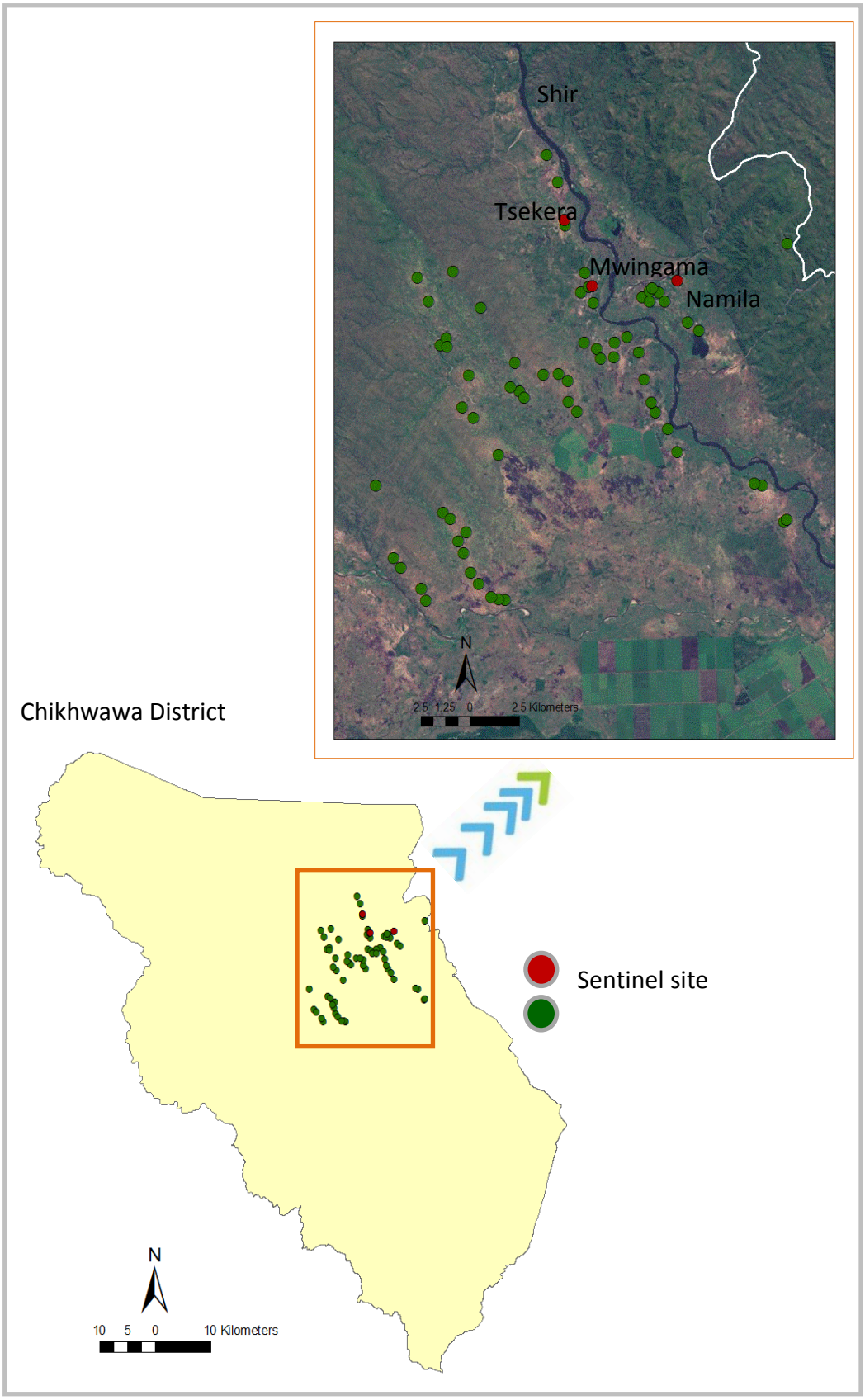

Figure 4.3 Map of Chikhwawa District showing the three sentinel sites and the 50 village catchment area for ACTia drug trial.

4.3.2. **Village description**

44.3.2.1 Mwingama Village

Mwingama village (16° 1' S, 34° 47' E) is located within the main town of the district and closest to the district referral hospital. It is about 2 kilometres from the Shire River and has combined modern and traditional households. The village has 2 main boreholes that provide water to the village and pools of water are found at these sites that potentially breed mosquitoes. In terms of agriculture, Mwingama has rice paddies; villagers grow cereals and vegetables and also rear goats, pigs, poultry and cattle. There are 112 households with a population of approximately 600 people. IRS was conducted on 12th February 2010 and all 6 households where window exit trap collections were ongoing were sprayed (Fig 4.4).

44.3.2.2 Namila Village

Namila (16° 0' S, 34° 49' E) is 15 kilometres northeast of Chikhwawa town centre. It has 277 households with a population of approximately 1200 people. The village is 10 kilometres away from the Shire River. It is close to a seasonal river called Likhubula, which dries up in dry season. Namila has traditional earth made houses thatched with grass and open eaves. The majority of homeowners are subsistence farmers rearing pigs, goats, poultry, and cattle. IRS was carried out on 28th February 2012 covering every household with an exit window trap (Fig. 4.4).

44.3.2.3 Tsekera village

Tsekera village (15° 59' S, 34° 46' E) is located to the southwest of the Chikhwawa town centre. The village is about 100 metres from the Shire River bed. It has 3 boreholes that provide off-river source of water and pools of water that can act as breeding sites for mosquitoes. The people are subsistence farmers, growing rice and cereals (maize, sorghum and millet) and many households rear pigs, goats, chickens and cattle. The village comprises of 60 households with a population of approximately 300 persons. The houses are typical traditional earth made with grass thatched roofs and open eaves. IRS was carried out on 17th February 2012. Every household with exit window trap was sprayed in Tsekera (Fig 4.4).

Fig 4.4. Arial view of Mwingama, Namila and Tsekera sentinel sites showing collection points within the village

4.3.3. **Assessment of Mosquito Species and Abundance**

Informed consent was obtained from homeowners who had shown interest to take part in the study (Appendix 1) and comprehensive training was provided to every household owner to show them how to undertake the daily mosquito collections. Safety information on handling of isopropanol, and other collection material as well as a practical illustration of the entire mosquito collection and recording process was conducted with a practice exercise to confirm understanding at every household. Refresher trainings and general update meetings were facilitated at each monthly visit to enhance the quality of the collection and identify and resolve potential problems encountered by the household owners in the course of the collection. A monthly stipend of $10 was given to each household owner as reimbursement for their time spent on collection. Window exit traps were installed in mid October 2010 on six houses from each sentinel site (18 traps in total) ahead of the rains and IRS intervention. Each household was provided with a tray of pre-labelled specimen jars containing isopropanol, a checklist form and an aspirator. Collection of mosquitoes was done daily at the following times; 6:00 a.m and 12:00 noon to ensure minimal escapees from the traps. Routine and surprise spot check visits were conducted for quality control purposes to check

whether the household owner was collecting the mosquito's and documenting the collections as per study operating procedures. Mosquitoes were then stored in the isopropanol specimen jars. Trays were retrieved monthly and household owners were asked to complete a checklist indicating the nights when the mosquitoes were collected [155]. Torn and worn out traps were reported and immediately replaced. All Culicines caught in the traps were recorded to ensure that in the absence of Anopheline catches, the traps were being operated successfully [46]. Mosquito specimens were sent to Chikhwawa District hospital laboratory for morphological species identification before being shipped to the Liverpool School of Tropical Medicine (LSTM) for species identification and sporozoite detection.

4.3.3.1. *Morphological species identification*

The collected mosquitoes were segregated into anophelines and culicine and enumerated at Chikhwawa District hospital laboratory. All anophelines were identified as *An. gambiae* complex or *An. funestus* group using morphological keys [55, 63] and individually stored on silica gel in micro-centrifuge tubes. Samples were then transported to LSTM for subsequent molecular analysis.

4.3.3.2. *Molecular Identification to species level*

Total genomic DNA (gDNA) was extracted from each mosquito head and thorax individually, using Qiagen DNeasy® 96 Blood and Tissue kit's according to manufacturer instruction, except with two amendments; At step 1 the mosquito tissue was disrupted using a QIAGEN Tissuelyser for 15 minutes at 30Hz after the addition of a stainless steel ball bearing, Buffer ATL and proteinase K to each collection tube. At step 16 and 17 the gDNA was eluted from the column using 50 µl only of elution buffer. Polymerase Chain Reaction (PCR) was then used to determine the species of each individual mosquito.

4.3.3.3. *Anopheles gambiae Complex*

Species identification was carried out using an adapted version of Scott *et al* [99]. A 25µl reaction was set up as follows; 1µl gDNA, 2.5µl 10X PCR Buffer, 0.25µl MgCl (50mM), 0.175µl Taq DNA polymerase, 0.5µl dNTPs (10mM), 18.24µl dH_20, 10mM primers of the following volumes; 0.624µl GA, 1.248µl UN, 0.463µl AR. PCR cycling conditions were set at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min and held at 10°C until taken off the PCR machine and stored at 4°C. The amplified fragments were analysed using 1.5% agarose gel containing ethidium bromide and visualised under UV light.

Primer name	Species name	Primer sequence (5' to 3')	Band size
GA	*An. gambiae s.s*	CTGGTTTGGTCGGCACGTTT	390
UN	-	GGTTGCCCCTTCCTCGATGT	-
ME	*An. melas/merus*	TGACCAACCCACTCCCTTGA	466
AR	*An. arabiensis*	AAGTGTCCTTCTCCATCCTA	315
QD	*An. quadriannulatus*	CAGACCAAGATGGTTAGTAT	153

Table 4.1 Primer sequences of species-diagnostic *An. gambiae* complex. GA = *An. gambiae s.s*, ME = *An. melas/merus*, AR = *An. arabiensis*, QD = *An. quadrianuulatus* and UN = Universal

4.3.3.3.1. Anopheles funestus Group

Anopheles funestus samples were species identified by PCR according to Koekemoer *et al* [215] and *An. funestus*-like according to Spilling *et al* [113]. Primers AFUN or MalaFB were used to amplify products diagnostic for *An. funestus s.s* (505 bp), and *An. funestus*-like (390 bp) respectively along with the internal transcribed spacer region 2 (ITS2A) primer. A 25 µl PCR reaction was then set up as follows; 1µl gDNA, 2.5µl 10X PCR Buffer, 1µl MgCl (50mM), 0.75µl Taq DNA polymerase, 2µl dNTPs (10mM), 0.5µl (10mM) of primers AFUN/MalaFB and ITS2A, and 16.75µl dH_20. Cycling conditions were set at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min and a hold at 10°C. Cycling conditions for *An funestus-like* identification were set as 94°C for 2 min's followed by 35 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 40 s and a final extension of 72°C for 5 min and a hold at 10°C.

PCR reactions were then stored at 4°C until the amplified fragments were analysed on 1.5% agarose gels containing ethidium bromide and visualised under UV light.

Primer name	Species name	Primer sequence (5' to 3')	Band size	Tm (^{0}C)
AFUN	*An. funestus s.s*	CGATCGATGGGTTAATC ATG	505	52.4
ITS2A	-	TGTGAACTGCAGGACAC AT	-	-
MalaFB	*An. funestus-like*	GTTTTCAATTGAATTCAC CATT	390	-

Table 4.2 Primer sequences of species-diagnostic An. funestus s.s, An. funestus-like and ITS2A (Universal) [215, 216].

4.3.3.4. *Sporozoite Detection*

Sporozoite detection was carried out on all gDNA samples of *An. gambiae*, *An. arabiensis*, *An. funestus s.s*, and *An. funestus*-like which were successfully identified using real time PCR (RT-PCR) as described by Bass *et al* [217]. Each reaction consisted of; 1µl gDNA, 10µl of 2x SensiMix probe (Bioline), 4.2µl dH_2O, 400nM final concentration of each probe by adding 0.8µl of 10µM probe PlasF+ (6FAM-TCTGAATACGAATGTC) and 0.8µl of 10µM OVM+ (VIC-CTGAATACAAATGCC), and 800nM final concentration of each primer by adding 1.6µl PlasF (GCTTAGTTACGATTAATAGGAGTAGCTTG) and 1.6µl PlasR (GAAAATCTAAGAATTTCACCTCTGACA). Samples were run on an Mx3005P

qPCR machine and the assay conditions were set as follows: 95°C for 10 min followed by 50 cycles of 95°C for 10 s and 60°C for 45 s. The increase in VIC and FAM fluorescence was measured at the end of each cycle on the yellow (530nm excitation and 555nm emission) and green channel (470 nm excitation and 510 emission) respectively. Positive samples were verified by analysing the amplification curves of samples relative to positive controls (MRA-273G, MRA-341G and a *P. falciparum* positive control gDNA sample) and with a cut-off point for the Ct value of 40 after, the threshold was set above background detection. Samples, which were positive between Ct values 40-45 were subjected to further testing and analysis for confirmation.

4.3.3.5. *Data analysis*

The numbers of mosquitoes per trap per night were calculated for each species based on day of capture of the specimen. Prevalence for specific species sporozoites and the number of infected mosquitoes per trap per night (transmission index) was determined. Sporozoite rates, number of mosquitoes per trap per 100 nights, transmission index and relative transmission index, percentage proportion of species and their estimated numbers were calculated using the following formulae:

- *Sporozoite rate* **=** the number of *Anopheles* infectious with sporozoites ÷ the total number tested for sporozoites.

- *Number of mosquitoes per trap per 100 nights* **=** (Total number of *An.* mosquitoes collected ÷ Total number of collection nights ÷ Total number of exit traps) x 100.
- *Transmission index* **=** Number of mosquitoes per trap per night x Sporozoite rate.
- *Relative transmission index* **=** Transmission Index ÷ Transmission index at base line
- *An. gambiae* s.s proportion (%) **=** (Total number of *An. gambiae* s.s ÷ Total number of *An. gambiae* s.l) x 100.
- *An. funestus* s.s proportion (%) **=** (Total number of *An. funestus* s.s ÷ Total number of *An. funestus* s.l) x 100

The changes in vector abundance and transmission parameters were assessed using Wilcoxon sign rank test.

4.3.4. **Assessment of Mosquito Insecticide Resistance**

Live collections were carried in randomly selected households within the three sentinel sites targeting indoor-resting adult female Anopheline mosquitoes pre and post IRS. Houses that had window exit traps were deliberately skipped for live collections to avoid confounding the abundance results. The first collections were carried out in December 2010 before the IRS intervention and then routinely after the spray.

Indoor resting blood fed adult female *An. gambiae s.l* and *An. funestus* were collected between 05.30-10.00 h in houses using a mouth aspirator. Live mosquitoes were then transported to Malaria Alert Centre insectary in Blantyre and kept in individual oviposition tubes with damp filter paper, provided with 10% sucrose, and allowed to lay eggs. Eggs were allowed to hatch in a small cup and latter transferred to bowls of water for rearing. The larvae were fed with Tetramin^{TM} baby fish food daily. Water in each larval bowl was changed every two days to reduce mortality due to poor water quality [158]. Each family was reared separately through to 1-3 day old F1 adults at 26°C +/- 2°C and 70-80% relative humidity. Families were mixed prior to testing to avoid bias from isofemale lines where offspring may all be genetically similar [218].

4.3.4.1 WHO Susceptibility Tests

Standard WHO susceptibility assay were carried out, exposing between 5 and 25, F1 3-5day old adult mosquitoes to insecticide treated, or control papers impregnated with the carrier oil alone, for 1 hour and then transferred to holding tubes with access to 10% sugar solution for 24 hours before the percentage mortality was determined. The insecticides tested were, bendiocarb (0.1%), deltamethrin (0.05%), etofenprox (0.5%), lambda-cyhalothrin (0.01%), malathion (5%), and permethrin (0.75%). Treated test papers with the WHO diagnostic dosages were supplied by

the WHO Collaborating Centre in Penang, Malaysia [156]. All dead mosquitoes post exposure to the insecticides were preserved in silica gel for molecular species identification, whilst the survivors were kept separately in RNAlater.

WHO insecticide resistance assay results were categorised according to percentage mortality as; susceptible, requiring confirmation of resistance, or resistant. This standard is recommended by WHO [219] and has been used by the African Network for Vector Resistance (ANVR) and has been adopted for the thesis (Table 4.3)

	At least 80 mosquitoes tested per bioassay	20 to 79 mosquitoes tested per bioassay
Susceptible	Mortality 98 – 100%	Mortality 98 – 100%
Resistance suspected, to be confirmed	Mortality 95 – 97%	Mortality 80 – 97%
Resistance	Mortality < 95%	Mortality <80%

Table 4.3: Criteria for interpretation and classification of results, based on WHO recommendations [219].

4.3.4.1. *Data Analysis for insecticide resistance*

Chi square test was used to compare insecticide susceptibility assay results over time from the same locality [154] and was calculated as follows:

- *Chi square:* $X^2 = \sum(O-E)^2/E$ = Sum of Number of [(observed number – expected number)^2] ÷ expected number.

  Where;

  X^2 = Sum of total number of $(O-E)^2/E$ computations,

  O = Observed number,

  E = Expected number.

Where the observed numbers were small (<5), Fischer's exact test was used to compare susceptibility assay results over time from the same locality.

4.3.5. **Assessment of Insecticide Quantification within Sprayed Households**

Post-spray insecticide concentrations on household walls were determined using a newly developed Insecticide Quantification Kit that used colorimetric analysis of insecticide content on felt wall pads according to Paine *et al* (Unpublished).

4.3.5.1. *Field Application of Insecticide Quantification Kits (IQK)*

Before the actual spraying, three felt pads were attached in duplicate to the walls of the same households that had exit window traps at each of three positions, top, middle and bottom. Details about the dates at which the wall pads were stuck or pulled off, household identification number and position were documented. Being small, the pads were less obvious to sprayers, who were not informed of the test and unaware of the location of the houses chosen or the purpose of the small pads. Each pad was 10mm diameter, 1mm thick and obtained from Game Stores, Chichiri, Malawi. One sample was taken in each house at random height at least three weeks after the spray to measure post-spray concentration and

then stored in a polythene bag at 4^{o}C in Blantyre, Malawi, before IQK analysis were done at LSTM, UK. Duplicate samples were taken for calorimetric analysis of insecticide content according to Paine *et al* (Unpublished).

4.3.5.2. *Insecticide Quantification*

For colorimetric measurement of lambda-cyhalothrin individual pads were dropped into a glass tube labeled with sample number. The IQK detection reagents were added to each tube as follows;

- 800 µL Reagent A (0.075% solution of potassium hydroxide in 90% ethanol)
- 800 µL Reagent B (400mg TTC and 40mg PNB, dissolved in 100ml with 90% ethanol)
- Incubate at RT for 15 min. with frequent mixing (vortexing if available)
- Add 400 µL reagent C (0.5ml of acetic acid in 100ml water; 0.5% final dilution).

A colour chart was prepared to compare observed levels by spiking pad size (1cm^{2}) 3mm filter papers with the active ingredient, lambda-cyhalothrin, as follows; a stock solution of lambda-cyhalothrin was prepared in 100% methanol. The filter papers were spiked with the following stock volumes; 1, 3, 5, 10, 20, 30 and 40 µl. A zero control was

prepared with 100 µl methanol. The filters were taken through the IQK procedure to provide the red colour range equivalent to wall spray rates of 0, 1, 2, 3, 5, 10, 20, and 40 mg/m^2 respectively. To produce the chart, the dilution range was transferred to clear plastic cuvettes (1 cm light path), and a picture taken against a white background. After importing into Powerpoint, representative red areas for each dilution were cropped and cut and pasted to produce a colour strip with depths of red colour representative of m^2 spray rates. Observed levels were compared visually against this colour strip for calorimetric analysis of insecticide content, to estimate the quantity of the residual insecticide of individual samples.

4.3.6. **Assessment of Human Burden Impact Indicators**

4.3.6.1. *The Rolling Malaria Indicator Surveys (rMIS)*

The rolling malaria indicator surveys (rMIS) were initiated in May 2010 and coordinated by the Malawi-Liverpool-Wellcome Trust (MLW) team based in Chikhwawa District Hospital. The MLW team was responsible for general management of the survey activities, including oversight of day-to-day operations, design of the survey and obtain ethical approval (both from the Liverpool School of Tropical Medicine Research Ethics Committee and College of Medicine Research Ethics Committee (COMREC), recruiting and training field staff, and providing necessary

medications for field activities. The MLW team also led the data processing activities, report writing and data dissemination.

The rMIS covered 50 villages in Chikhwawa district (~1/10 of the entire district) including the sentinel sites of this study (Fig 4.3). The design for the survey was a representative probability sample to produce estimates for the study area as a whole and each season separately. To assess differences between the rainy season from November to April, and dry season from May to October, every village was sampled once in each six-month period (once during the rainy season and once during the dry season). On average, a six-month gap between first and second sample for each village was ensured to avoid treatment interference on first survey with burden assessments on the second survey.

Households were randomly selected using a two-step sampling strategy. During each season, all 50 villages were randomly assigned to one of the six months (8 or 9 villages per month). Within each village, households were randomly selected from a list of households, with a probability proportional to village population size. This sampling frame was based on a research-driven census exercise of the entire study area conducted by the study team in November 2008.

4.3.6.2. *Malaria parasites and anaemia testing*

All nurses recruited for the rolling MIS received standardized training to conduct finger pricks for anaemia and malaria parasitaemia among children six months to 59 months. Blood samples were collected to prepare a thick and thin blood film, determine the child's haemoglobin concentration using a Hemocue photometer (HemoCue Haemoglobin 301 analyser, Hemocue Ltd, UK), and guiding treatment of parasitaemic children in the field by conducting a rapid malaria-diagnostic test (RDT) strip (First Response Malaria Ag pLDH/HRP2 Combo, Premier Medical Corporation Ltd. India) . Results from the anaemia testing and RDTs were available immediately to the parents or caregivers for the child. Children with a positive RDT and without clinical evidence for severe malaria classification received treatment on the spot for malaria using Coartem® from study research nurses, according to Malawi national treatment guidelines. Children clinically assessed by the survey nurse to need further medical assessment and care were referred and assisted with transport to the Chikhwawa District Hospital. Those already treated with Coartem® within the past two weeks were also referred to Chikhwawa District Hospital for additional evaluation.

4.3.6.3. *Slide examination*

Children found to be parasitaemic (by RDT) or anaemic were treated as per national malaria treatment guidelines. Questionnaires were pre-programmed into the PDAs (Somo 650®, Socket Mobile, Newark, California) programmed in Visual CE® 11.1 language (Syware Incorporation, Cambridge, Massachusetts) to eliminate the need for paper-based questionnaires. Both, Hemocue (HemoCue B-Hemoglobin®, HemoCue AB, Ängelhom, Sweden) and RDT (First Response® Malaria Ag. pLDH/HRP2 Combo Card Test, Premier Medical Corporation Ltd., India) testing were performed according to manufacturer recommendations.

4.4. **Ethical approval**

The Research Ethics Committees of the College of Medicine in Blantyre (COMREC) (refs P.08/10/970 and P.10/08/707) and the Liverpool School of Tropical Medicine (refs 09.07 and 10.78) provided ethical approval for the ACTia main study. Permission to work in specific villages was granted by each village chief following an initial briefing meeting at which the nature, objectives of the study were explained to all members of the community in local language, Chichewa. Written informed consent was obtained at the beginning of the study. On the day of live mosquito collection, the purpose of the work was again explained to each householder, and permission to enter the house was sought.

5. RESULTS

5.1. Vector Abundance and Transmission indicators

5.1.1. Vector Species Identification

A total of 3395 Anophelines were collected from 18 window exit traps over a 577 trapping night period (October, 2010 to April 2012). Of these, 607 (18%) were collected from Mwingama, 270 (8%) from Namila, and 2518 (74%) were collected from Tsekera. From the total *Anophelines* collected, 1715 were morphologically identified as *An. gambiae s.l* (50.5%) and 1680 were identified as *An. funestus s.l* (49.5%). Of these, 1396 *An. gambiae s.l* and 1063 *An. funestus s.l* were further identified to species level using PCR (Tables 5.1, 5.2 and 5.3). *Anopheles gambiae s.s* and *An. arabiensis* were the two members of the *An. gambiae* complex identified and *An. funestus s.s* was the only member of *An. funestus* group identified, all three species were found at all three sites (Tables 5.1, 5.2 and 5.3).

5.1.2. Vector Species Abundance

In the period leading up to IRS, October 2010 to February 2011, the total number of *An. gambiae s.l* and *An. funestus s.l* caught from Mwingama was 228 and 187 respectively. From this, the calculated number of *An.*

gambiae s.s, *An. arabiensis* and *An. funestus s.s* caught per window trap per 100 nights was 0.35, 24.3 and 17.6 respectively (Table 5.3). Following IRS, March 2011 to July 2011, there was a decline in mosquitoes being caught, with only 6 *An. gambiae* using Wilcoxon test ($z = 0.97$, $p = 0.33$) and 19 *An. funestus* ($z = 3.2$, $p = 0.75$). The calculated number of *An. gambiae s.s*, *An. arabiensis* and *An. funestus s.s* caught per window trap per 100 nights for Mwingama was 0.13, 0.53 and 1.51 respectively. Normally there would be a natural reduction in the mosquito population at this time, as the rains would decline, however, the peak rainfall was later than expected for the malaria season of 2010/11 (Fig 5.1). Decline in abundance are best observed when comparing two comparable seasons, i.e., October 2011 to Feb 2012, where a decline is observed, 14 *An. gambiae* ($z = 0.74$, $p = 0.46$) and 6 *An. funestus* ($z = 0.21$, $p = 0.83$) despite a higher rainfall in the second season and an expected increase higher abundance. Culicines were collected in window exit traps suggesting that these were still well operated throughout the study. Fig 5.1 summarises the comparisons of the mosquito numbers caught per village before and after IRS.

A decline in *An. gambiae* and *An. funestus* also occurred in Namila following IRS. The number of *An. gambiae s.l* and *An. funestus s.l*, from October 2010 to February 2011, was 70 and 76 respectively (Fig 5.2). The

calculated number of *An. gambiae s.s*, *An. arabiensis* and *An. funestus s.s* caught per window trap per 100 nights was 0, 6.33 and 5.40 respectively. Following IRS, March 2011 to July 2011, there was a significant decline in mosquitoes being caught with only 5 *An. gambiae* ($z = 2.4$, $p = 0.02$) and 26 *An. funestus*. The number of *An. gambiae s.s*, *An. arabiensis* and *An. funestus s.s* caught per window trap per 100 nights for Namila over this period dropped to 0, 0.44 and 0.85 respectively. The decline in abundance for this site was observed when comparing pre IRS with mosquito abundance of the corresponding season, of October 2011 to Feb 2012, where significant decline in *An. gambiae* ($n = 16$, $p = 0.05$) and *An. funestus* ($n = 3$, $p = 0.17$) (Fig 5.2).

Mosquito decline was also observed at Tsekera where the number of *An. gambiae s.l* (1097), and *An. funestus s.l* (766) caught before IRS dropped to 187 *An. gambiae* ($p = 0.35$) and 233 *An. funestus* ($p = 0.92$) from March to July 2011. The calculated number of *An. gambiae s.s*, *An. arabiensis* and *An. funestus s.s* caught per window trap per 100 nights was 3.74, 117 and 63.9 respectively (Table 5.4). Despite a higher rainfall in the second season and an expected increase higher abundance, only 1 *An. gambiae s.l* ($p = 0.07$) and 1 *An. funestus s.l* ($p = 0.17$) was collected at Tsekera from October 2011 to February 2012.

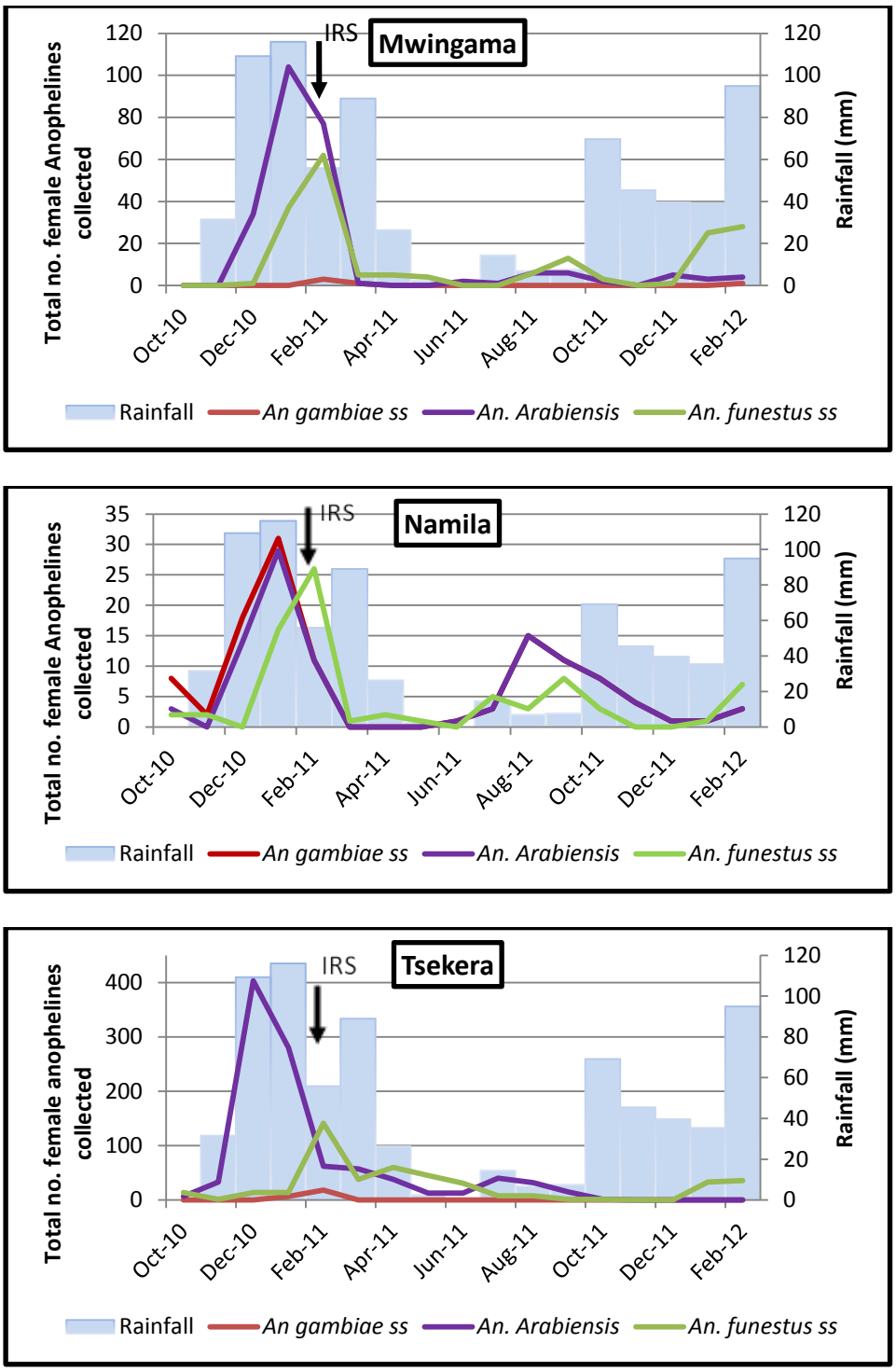

Fig 5.1 *Anopheles* mosquito species abundance in the three sentinel sites and district level monthly rainfall distribution.

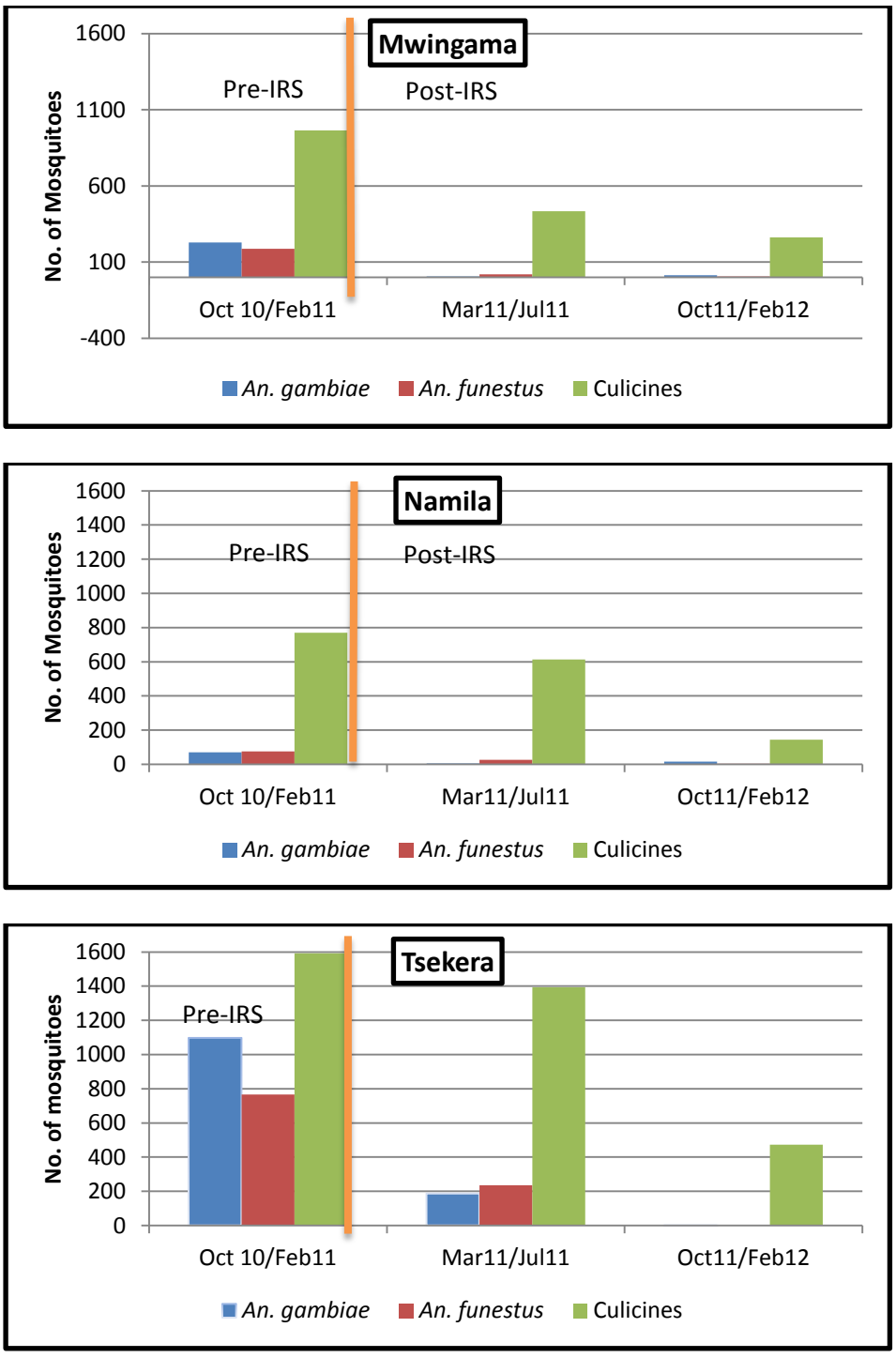

Fig 5.2 *Anopheline* and non *Anopheline* mosquito abundance in the sentinel sites pre and post IRS.

Table 5.1 Vector Abundance, Infectivity and Transmission Index for Mwingama Pre and Post IRS Intervention			
	Pre IRS	Post IRS	
	Oct 2010 – Feb 2011	Mar - July 2011	Oct 2011 – Feb 012
***An. gambiae* s.l**			
No. Caught	228	6	14
No. Analysed for species id	219	5	14
No. *An. gambiae* s.s	3	1	0
No. *An. arabiensis*	215	4	14
No. Others	1	0	0
No. *An. gambiae* s.s proportion (%)	1.32	20	0
***An. gambiae* s.s**			
No. Estimated	3.12	1.2	0
No. *An. gambiae* s.s per trap per 100 nights	0.34	0.13	0
Sporozoite Rate	0 (n=3)	0 (n=1)	0 (n=0)
Transmission Index	0	0	0
An. arabiensis			
No. Estimated	224	4.8	14
No. *An. gambiae* s.s per trap per 100 nights	25	0.53	1.57
Sporozoite Rate	0.03 (n=215)	0 (n=4)	0.07 (n=14)
Transmission Index	0.92	0	0.11
No. *An. arabiensis* proportion (%)	98.2	0.8	100
***An. funestus* s.l**			
No. Caught	187	19	6
No. Analysed for species id	117	16	6
No. *An. funestus* s.s	100	14	5
No. *An. funestus* proportion (%)	85.5	87.5	83.3
***An. funestus* s.s**			
No. Estimated	160	16.6	5
No. per trap per 100 nights	18	1.51	0.56
Sporozoite rate	0.02 (n=117)	0 (n=17)	0.17 (n=6)
Transmission Index	0.3	0	0.1

Table 5.2 Vector Abundance, Infectivity and Transmission Index for Namila Pre and Post IRS Intervention			
	Pre IRS	Post IRS	
	Oct 2010 – Feb 2011	Mar - July 2011	Oct 2011 – Feb 012
An. gambiae s.l			
No. Caught	70	5	16
No. Analysed for species id	70	5	16
No. *An. gambiae* s.s	0	0	0
No. *An. arabiensis*	57	4	16
No. Others	13	1	0
No. *An. gambiae* s.s proportion (%)	0	0	0
An. gambiae s.s			
No. Estimated	0	0	0
No. *An. gambiae* s.s per trap per 100 nights	0	0	0
Sporozoite Rate	0 (n=0)	0 (n=0)	0 (n=0)
Transmission Index*	0	0	0
An. Arabiensis			
No. Estimated	57	4	16
No. *An. gambiae* s.s per trap per 100 nights	6.3	0.44	1.79
Sporozoite Rate	0 (n=57)	0 (n=4)	0.07 (n=16)
Transmission Index*	0	0	0.11
No. *An. arabiensis* proportion (%)	8.4	0.8	100
An. funestus s.l			
No. Caught	76	26	3
No. Analysed for species id	72	25	3
No. *An. funestus* s.s	46	9	3
No. *An. funestus* proportion (%)	63.9	36	100
An. funestus s.s			
No. Estimated	48.6	9.4	3
No. per trap per 100 nights	5.4	0.85	0.34
Sporozoite rate	0.03 (n=72)	0 (n=25)	0.17 (n=3)
Transmission Index*	0.15	0	0.11

Table 5.3 Vector Abundance, Infectivity and Transmission Index for Tsekera Pre and Post IRS Intervention			
	Pre IRS	Post IRS	
	Oct 2010 – Feb 2011	Mar - July 2011	Oct 2011 – Feb 012
An. gambiae s.l			
No. Caught	1097	187	1
No. Analysed for species id	815	161	1
No. *An. gambiae* s.s	25	0	1
No. *An. arabiensis*	785	161	1
No. Others	5	0	0
No. *An. gambiae* s.s proportion (%)	3.07	0	0
An. gambiae s.s			
No. Estimated	3.4	0	0
No. *An. gambiae* s.s per trap per 100 nights	3.74	0	0
Sporozoite Rate	0.08 (n=25)	0 (n=0)	0 (n=0)
Transmission Index*	0.03	0	3
An. Arabiensis			
No. Estimated	1057	187	1
No. *An. gambiae* s.s per trap per 100 nights	117.4	20.6	0.01
Sporozoite Rate	0.02 (n=785)	0.02 (n=161)	0 (n=1)
Transmission Index*	1.94	0.51	0
No. *An. arabiensis* proportion (%)	96	1	100
An. fune3stus s.l			
No. Caught	766	236	1
No. Analysed for species id	245	207	1
No. *An. funestus* s.s	184	182	1
No. *An. funestus* proportion (%)	75.1	87.9	100
An. funestus s.s			
No. Estimated	575	207.5	1
No. per trap per 100 nights	63.9	18.9	0.1
Sporozoite rate	0.01 (n=245)	0.09 (n=207)	0 (n=1)
Transmission Index*	4.17	1.64	0.11

	An. gambiae		*An. arabiensis*		*An. funestus*	
Mwingama	Mosquitoes per trap per 100 days, (n)	95% CI	Mosquitoes per trap per 100 days, (n)	95% CI	Mosquitoes per trap per 100 days, (n)	95% CI
Pre-IRS	0.34 (3)	[0.1-4.2]	25 (215)	[11.6-22.1]	18 (117)	[9.3-25.2]
Post-IRS	0.13 (1)	[0.1-5.2]	0.53 (4)	[0.1-5.6]	1.51 (17)	[0.2-7.1]
Namila						
Pre-IRS	0 (0)	-	6.3 (57)	[3.2-11.4]	5.4 (72)	[2.4-11.2]
Post-IRS	0 (0)	-	0.44 (4)	[0.1-6.3]	0.85 (25)	[0.1-6.4]
Tsekera						
Pre-IRS	3.74 (25)	[0.9-12.4]	117.4 (785)	[99.2-131.7]	63.9 (245)	[48.4-73.1]
Post-IRS	0 (0)	-	20.6 (161)	[11.5-29.4]	18.9 (207)	[12.3-31.1]

Table 5.4 Mosquito collections per trap per 100 days from the sentinel sites

5.1.3. **Sporozoite Rates**

Sporozoite rates were determined in all the identified malaria vector species. In the period October 2010 to February 2011 The *An. funestus s.s* sporozoite rate for Mwingama, Namila, and Tsekera was 2%, 4% and 8% respectively. During the post IRS period this was 0 at Mwingama and Namila (P = 0.137) and 8% at Tsekera from March to July 2011 (Tables 5.1, 5.2 and 5.3).

As no *An. gambiae* were found between Oct 2010 and Feb 2011 during the pre-IRS assessment, no sporozoite rates could be determined for *An. gambiae* at Mwingama and Namila pre IRS, whilst that of Tsekera was

0.08%. There was no *An. gambiae* collected in the period October 2011 to February 2012, and therefore no sporozoite was detected.

5.1.4. **Transmission Index**

The transmission index is defined as the average number of female *Anopheles* found with sporozoite in the salivary glands per room per day. Transmission index for *An. gambiae* (all sites combined) was 0.003 before IRS and 0 in the following time period from October 2011 to February 2012. *An. funestus* transmission index declined from 3.2 before IRS to 0.1 over the period October 2011 and February 2012.

Transmission index was recorded when the sites were taken individually. *Anopheles funestus s.s* transmission index, pre IRS, was 0.3 for Mwingama, 0.1 for Namila and 4.17 for Tsekera. Following IRS, from February to July 2011, the transmission index fell to 0 at both Mwingama and Namila and 1.64 at Tsekera (Tables 5.1, 5.2 and 5.3).

5.2. **Insecticide Resistance**

A total of 2213 *Anopheles* were obtained from the indoor live catches in all the three sentinel sites between from October 2010 to February 2012, of which 199 (9%) were *An. gambiae s.l* and 2014 (91%) were *An. funestus*

s.l. Of these 67 *An. gambiae* and 302 *An. funestus* laid eggs that were reared to 1-3 day old F1 generation and assayed for insecticide susceptibility using WHO protocol. A total of 695 *An. gambiae s.l* and 2516 *An. funestus s.l* were assayed.

Insecticide resistance status was based on percentage mortality. Based on the WHO definition, clear insecticide resistance was only found to deltamethrin in *An. funestus* (77% mortality) from Namila in 2011 (Table 5.4). Tests on all other insecticides showed suspected resistance in both species of mosquitoes except at Mwingama where *An. funestus* (99% mortality in 2011) and *An. gambiae* (98% mortality in 2012) showed clear susceptibility to etnofenprox. *An. funestus* was also fully susceptible to malathion in 2011 (98% and 99% mortalities) at Mwingama and Tsekera respectively.

A comparison of resistance status of *An. gambiae s.l* and *An. funestus s.l* between the two years (2011 and 2012) against different insecticides was determined. There was a significant change in resistance pattern at Namila in *An. funestus* to lambda-cyhalothrin between 2011 and 2012 (X^2 = 6.011, P = 0.014). *Anopheles funestus* results from Namila showed no significant change to permethrin (91% mortality in 2011 and 87% mortality in 2012) (X^2 = 0.433, P = 0.611). No significant change in

resistance was found to a pseudo-pyrethroid, etofenprox was shown at Namila between 2011 and 2012 (X^2 = 0.147, P = 1.000).

Similarly, there was no significant change in the percentage mortality for *An. funestus* to deltamethrin at Mwingama between 2011 (88% mortality) and 2012 (83% mortality) (X^2 = 0.030, P = 1.000). Results show low cross resistance detected in both *An. funestus s.l* and *An. gambiae s.l* to carbamate and organophosphate insecticides.

Very few tests were carried out on *An. gambiae s.l* from all the three sites. This was due to very low numbers of indoor resting *An. gambiae* being found and subsequently caught at the sites. Results for the tests on *An. gambiae s.l* from Namila showed a suspected level of resistance to lambda-cyhalothrin (84% mortality) in 2012. There was low resistance of *An. gambiae s.l* from Namila to malathion (94% mortality) in 2012 and bendiocarb (95% mortality) from Tsekera in 2012.

	Year 2011 / Site	Bendiocarb (0.01%)			Deltamethrin (0.05%)			Lambda-cyhalothrin (0.75%)			Permethrin (0.75%)			Malathion (5%)			Etnofenprox (5%)		
An.funestus		N	n_d	%M	n	n_d	%M	N	n_d	%M	n	n_d	%M	n	n_d	%M	n	n_d	%M
	Sekera										76	64	84	78	77	99	82	75	91
	Mwingama				86	78	88							74	73	98	77	76	99
	Namila	79	77	97	69	53	77	135	111	82	75	68	91				78	76	97
An.gambiae																			
	Sekera							82	71	87									
	Mwingama																		
	Namila	44	41	91															

	Year 2012 / Site	Bendiocarb (0.01%)			Deltamethrin (0.05%)			Lambda-cyhalothrin (0.75%)			Permethrin (0.75%)			Malathion (5%)			Etnofenprox (5%)		
An.funestus		N	n_d	%M	n	n_d	%M	N	n_d	%M	n	n_d	%M	n	n_d	%M	n	n_d	%M
	Sekera	81	75	93	171	149	87				81	73	90	80	77	96	82	79	96
	Mwingama	153	145	95	241	199	83	144	119	83	89	77	87	73	71	97			
	Namila	82	79	96				82	77	94	79	69	87	86	82	95	83	80	96
An.gambiae																			
	Sekera	80	76	95				86	72	84									
	Mwingama										86	74	86				80	78	98
	Namila				80	73	91				75	68	91	82	77	94			

Table 5.5 WHO bioassay results for years 2011 and 2012 (**n** = total number of mosquitoes tested; **n_d** = number dead; **% M** = Percentage-mortality). Note; The percentage control mortalities for all the bioassays were between 99% and 100%.

5.3. **Insecticide Quantification**

Every household with a window exit trap was sprayed with alpha-cypermethrin. This was not mandatory as household owners had a choice to accept or decline the spraying activity. Before the spray round started, nine pads were stuck randomly on the walls with 3 at high height, above 6 foot, 3 at medium height, between 3 – 6 foot and 3 at low height, less than 3 foot. At least two weeks after the spraying a pad from each height from each household was removed and tested by insecticide quantification kit (IQK) with reference to a colour chart (Fig 5.3). A total of 32 pads were screened for insecticide content. Individual readings were taken to estimate variability of spray quality in each house. In order to estimate the average spray quality of each house, the individual reactions were pooled and the colour measured to provide a 'pooled average'. Results of insecticide quantities and corresponding colorimetric images are listed in Table 5.5, Figures 5.2 – 5.9.

LSTM code	Date of Spray	Date of Collection	Time on wall	Village	Wall Type	House	Wall Position	1/2 Pad µg (2nd)	Full Pad µg/cm2 (2nd)	mg/m2 (2nd)	1/2 Pad mg/m2 (1st)	Full Pad mg/m2 (1st)
1414	17/02/11	3/24/2011	5 Weeks	Tsekera	Earth Wall	HH4	Lower	0.25	0.5	5		
1415	17/02/11	3/24/2011	5 Weeks	Tsekera	Earth Wall	HH4	Upper	0	0	0		
1416	17/02/11	3/24/2011	5 Weeks	Tsekera	Burnt Brick	HH5	Middle	0	0	0	3.9	7.8
1417	17/02/11	3/24/2011	5 Weeks	Tsekera	Burnt Brick	HH5	Upper	0	0	0		
1418	17/02/11	3/24/2011	5 Weeks	Tsekera	Burnt Brick	HH6	Lower					
1419	17/02/11	3/24/2011	5 Weeks	Tsekera	Burnt Brick	HH6	Upper	0.25	0.5	5		
1420	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH1	Middle	1	2	20	62.5	125
1421	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH1	Upper	0.5	1	10		
1422	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH2	Lower	0.25	0.5	5		
1423	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH2	Upper	0	0	0		
1424	12/2/2011	3/25/2011	5 Weeks	Mwingama	Earth Wall	HH3	Middle	0.5	1	10	31.25	62.5
1425	12/2/2011	3/25/2011	5 Weeks	Mwingama	Earth Wall	HH3	Upper	1	2	20		
1426	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH4	Lower	0.25	0.5	5		
1427	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH4	Middle	0.5	1	10	31.25	62.5
1428	12/2/2011	3/25/2011	5 Weeks	Mwingama	Earth Wall	HH5	Lower	0	0	0		
1429	12/2/2011	3/25/2011	5 Weeks	Mwingama	Earth Wall	HH5	Upper	0	0	0		
1430	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH6	Middle	0	0	0		
1431	12/2/2011	3/25/2011	5 Weeks	Mwingama	Burnt Brick	HH6	Upper	0	0	0		
1432	28/02/11	3/31/2011	4 weeks	Namila	Earth Wall	HH1	-	1	2	20	250+	250+
1433	28/02/11	3/31/2011	4 weeks	Namila	Earth Wall	HH1	-	2	4	40		
1434	28/02/11	3/31/2011	4 weeks	Namila	Earth Wall	HH2	-	0	0	0		
1435	28/02/11	3/31/2011	4 weeks	Namila	Earth Wall	HH2	-	0	0	0		
1436	28/02/11	3/31/2011	4 weeks	Namila	Earth Wall	HH3	-	0	0	0		
1437	28/02/11	3/31/2011	4 weeks	Namila	Earth Wall	HH3	-	0	0	0		
1438	28/02/11	3/31/2011	4 weeks	Namila	Burnt Brick	HH5	-	0	0	0		
1439	28/02/11	3/31/2011	4 weeks	Namila	Burnt Brick	HH5	-	0	0	0		

Table 5.6 Summary of results of wall pads and corresponding household attributes. **Note:** Empty entries mean there are no data present due to different reasons. ½ Pad µg concentrations are those collected from immersion reagents in half the wall pad.

Fig 5.3 Standard alpha-cypermethrin serial dilutions.

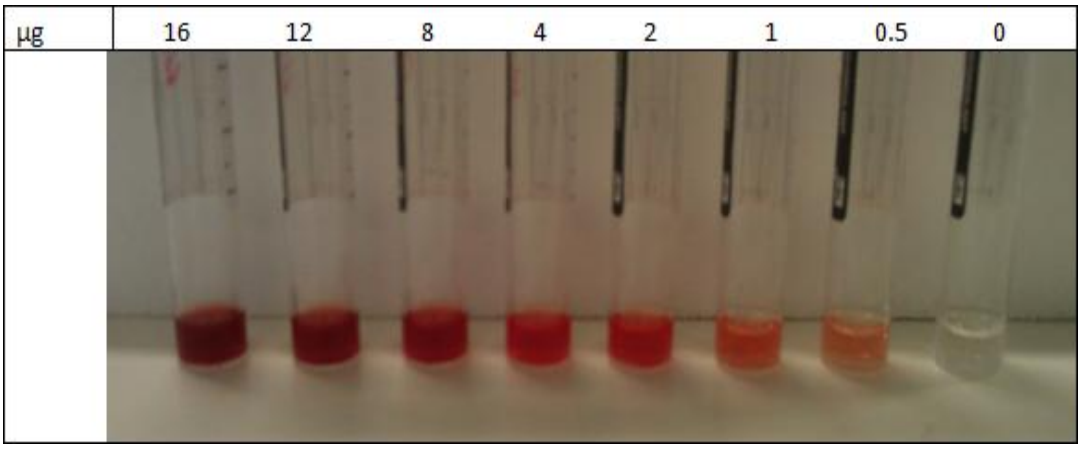

Fig 5.4 Tsekera wall pads

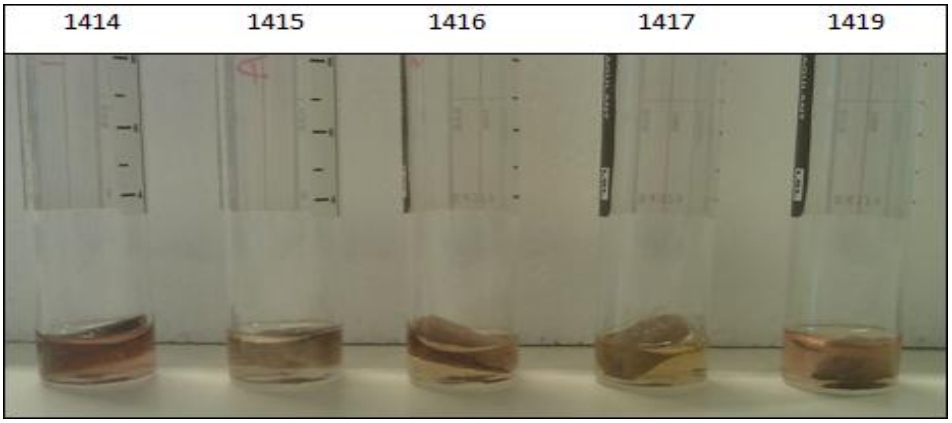

Fig 5.5 Mwingama wall pads

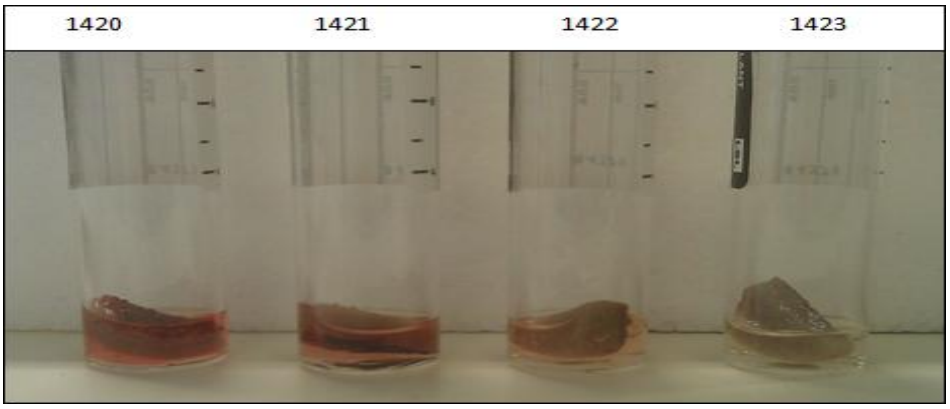

Figure 5.3; Standard Alpha-cypermethrin serial dilutions made in methanol. Left to right: 16, 12, 8, 4, 2, 1, 0.5 and 0 mg/m^2. **Fig 5.4;** Calorimetric results for Sekera wall pad samples 1414-1419. **Fig 5.5;** Results for Mwingama samples 1420-1423. Insecticide was extracted from half a pad using acetone, then 5µl inoculated into the colorimetric reagents.

Fig 5.6 Mwingama wall pads

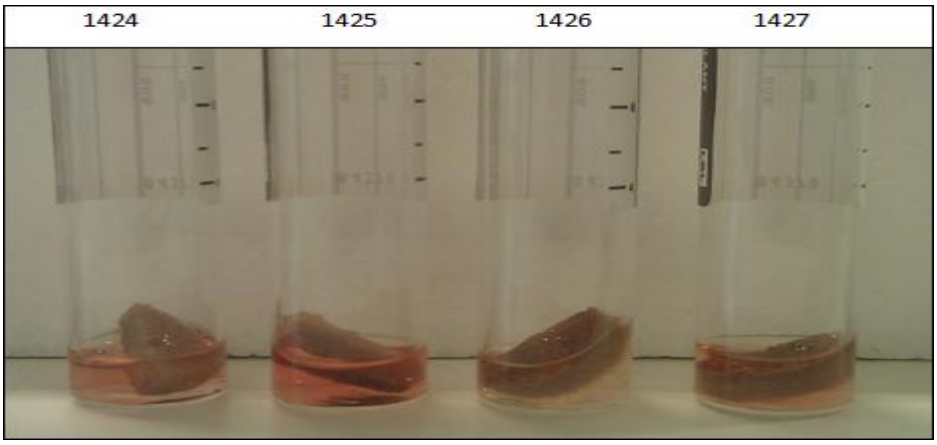

Fig 5.7 Namila wall pads

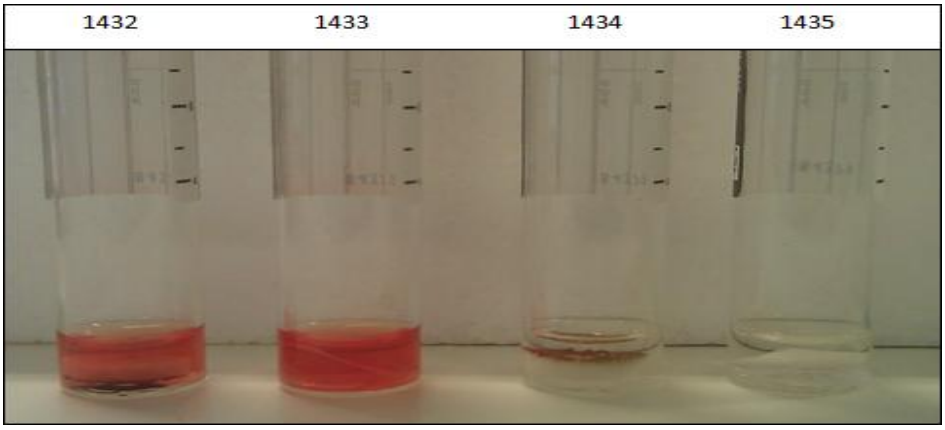

Fig 5.8 Namila wall pads

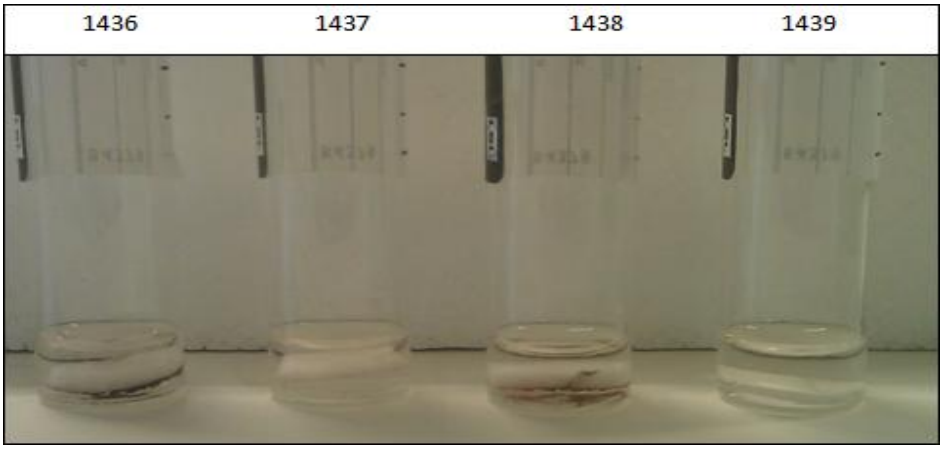

Fig 5.6; Colorimetric results for Sekera wall pad samples 1424-27. **Fig 5.7;** Colorimetric results for Namila samples 1432-35. **Fig 5.8** Colourmetric results for Namila samples 1436-39. Insecticide extracted from half a pad using acetone, then 5µl inoculated into the colorimetric reagents.

5.4. **Malaria and anaemia prevalence in the study site**

A continuous, rolling Malaria Indicator Survey (rMIS), was carried out from May 2010 to April 2011 covering a total of 637 young children of 6-59 months old. This was developed into an expanded malaria indicator survey (eMIS), which was conducted from May 2011 to date, as a continuation to rMIS, but involving participants of all ages. A total of 1276 households were enrolled in which 637 (28%) were children 6-59 months old.

The reported household coverage of ITN and IRS is shown across the entire catchment area (Figure 5.9). During the 12 months post IRS period (March 2011 to March 2012), the reported average household IRS coverage for the 50 villages was 63% on average, declining sharply in April 2012 after expiry of the effective 12 month post-campaign period. Household Insecticide treated net coverage for the entire catchment area did not change significantly during the pre and post –IRS period

The seasonal variation in *P. falciparum* parasitaemia prevalence across the entire 50 village catchment area before and after IRS is summarised in Figure 5.10 Results show parasitaemia prevalence declining from an average of 41% in the year before IRS, to 19% in the year following IRS, while moderate to severe anaemia (Hb< 8 g/dL) prevalence dropped to almost zero post IRS.

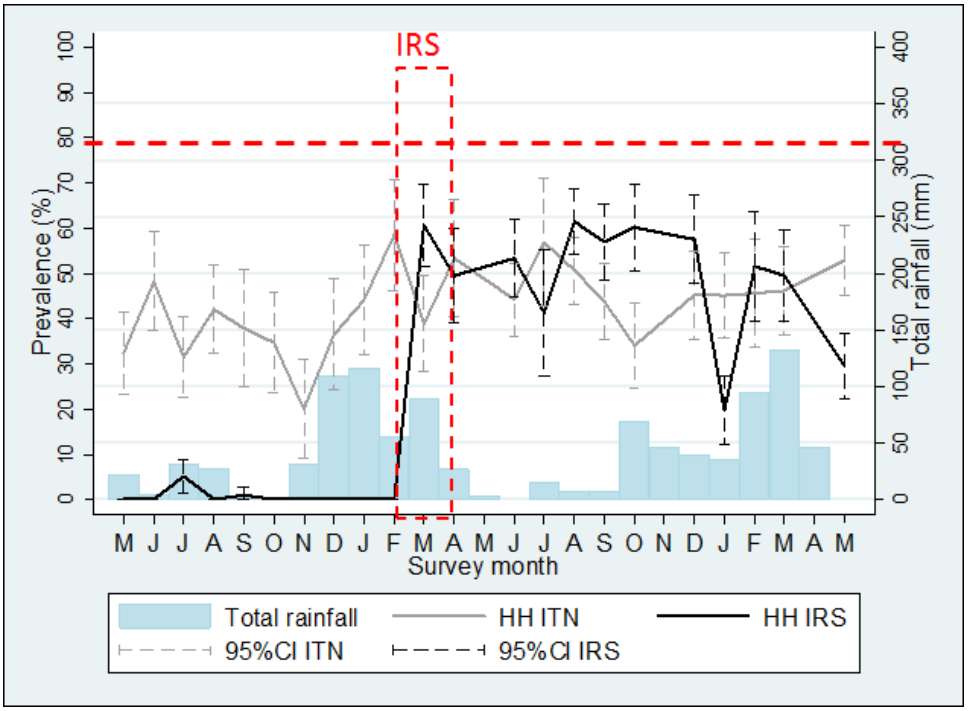

Figure 5.9: Intervention coverage by ITN and IRS for the 50 village catchment area from 2010 to 2012

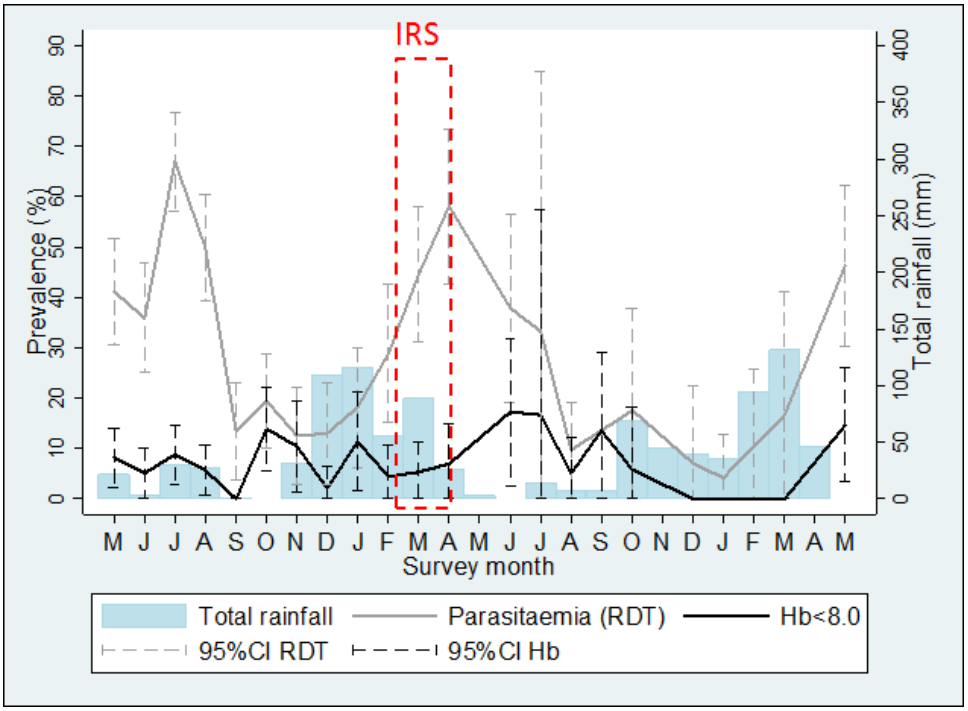

Figure 5.10: parasite and anaemia prevalence, and rainfall for the overall study site from May 2010 to May 2012

6. DISCUSSION

This work originated on the premise that direct monitoring and assessment of mosquito abundance and malaria transmission could measure the impact of vector control. The study incorporated field, laboratory and insectary work to monitor the insecticide resistance status of *An. gambiae* and *An. funestus;* the predominant malaria vectors in Chikhwawa. The study also aimed to increase our knowledge of the malaria vectors in general to inform the scaling-up of indoor residual spraying in Malawi. While repeated rounds of IRS were planned, the National Malaria Control Programme implemented only a single IRS round during the study period. This provided an opportunity to assess the change in entomological indicators over time and duration of a standalone IRS round on top of other interventions.

6.1. Main Findings

6.1.1. Mosquito Abundance and Disease Transmission

This study found a large heterogeneity in *An. gambiae* and *An. funestus* abundance between these nearby sentinel sites. Seventy four percent of the entire window exit trap collections were from Tsekera while only 18% was from Mwingama and 8% from Namila. The reasons for this heterogeneity in abundance, is likely multifactorial, linked to actual differences in breeding sites (some sites were close and the other further from the river) and/or factors that affected our measurement of abundance for instance the preferential choice of non-open eaves houses.

Tsekera village includes a higher number of water pools, this site was closest to the Shire River and with a lot of irrigation. Little irrigation was done at Namila and Mwingama, and Mwingama is an urban centre among the sentinel sites. Our quantification method of the water pools was based on visual inspection, scoring and counting informally at the time of the selection of suitable sites (not presented), rather than a standardized quantification technique to quantify breeding sites in order to properly explain the variations in mosquito abundance between households and villages on the basis of water pools. While the use of a well defined methodology such as that described by Sattler *et al* [220-222]) would have improved our ability to define this association, the observed data on water sources does suggest a strong heterogeneity in breeding sites on a fine scale.

Housing structure can be a main determinant of the number of mosquito's present in a household. Mwingama was the only urban site, located within Chikhwawa town centre, where the majority of houses are iron roofed and burnt bricked. Mosquito house entry has been shown to be reduced through simple changes in house design [223, 224] such as closing eaves, installing a ceiling, screening external doors and windows and a general improvement in quality of construction materials [224]. In these instances house entry rates are probably reduced by physically blocking or decreasing the number of holes through which a mosquito may gain access to a home. Houses can also be made less suitable for indoor resting mosquitoes by making them well lit, with few places for adult vectors to

rest, and this is often cited as one of the reasons for the decline in malaria in Europe [225].

One surprising observation from the study was that the numbers of both *An. gambiae* and *An. funestus* in the season November 2011/March 2012 remained low in all the three sentinel sites despite the fact that IRS was not implemented that season. This is against a background of comparatively higher rainfall received in that season than the previous one. In Malawi the rains normally begin late October and this leads to a steady increase in mosquito population that peaks in January and then declines to low levels again by the end of the rain in April. This would, all else being equal, result in a higher abundance in the 2011/12 season than 2010/11. More so, *An. gambiae s.s* thrives and predominates in humid conditions whilst *An. funestus* larvae thrive in grassy edges or shaded area of permanent and semi-permanent water bodies [55, 63], typical of Chikhwawa, and therefore we expected to observe increase in numbers of both species at this point. Obvious concerns of quality assurance as an attribute to such lower numbers collected over the period were ruled out by the fact that *Culicines* and flies were still collected in window exit traps suggesting that these were still being emptied on a daily basis as per agreed study procedures, this was confirmed at the time of unannounced spot check visits.

There was a drop *An. funestus* sporozoite rate for Mwingama, Namila, and Tsekera from 2%, 4% and 8% respectively to 0 between February and July 2011 post IRS. This is technically the number of *Anopheles* infectious with

sporozoites divided by the total number of *An.* tested for sporozoites. This result is in agreement with trends in parasitaemia prevalence obtained in under 5yrs old children within the 50 village area which also dropped from about 55% to 12% from April to August 2011 (Fig. 5.1). The drop in parasitaemia could be explained by an increase in multifaceted intervention coverage by ITN and IRS for the 50 village catchment area from 2010 to 2012 in addition to adoption of policies that is promoting confirmed malaria diagnosis using malaria microscopy and / or rapid diagnostic tests (RDTs) from 2012. This could reduce parasite recrudescence.

6.1.2. **Insecticide Resistance**

A study in Chikhwawa district in 2007 detected no resistance to pyrethroids in *An. gambiae*, *An. funestus* and *An. quadrianulatus* [211]. However, we report here just years later the results of WHO bioassays that detected low (suspected) levels of insecticide resistance in both *An. gambiae* and *An. funestus* to the pyrethroids deltramethrin, lambda-cyhalothrin and permethrin. This suggests a selection for insecticide resistance in the last 4-5 years. The levels of pyrethroid resistance found here are in agreement with the results found in a 2012 study where the authors showed elevated levels of p450s are associated with the pyrethroid resistance in Chikhwawa *An. funestus* [157].

This same resistance pattern in *An. funestus* populations has been reported in southern [152, 226, 227] and northern Mozambique [228]; close to the Malawi border. More recently evidence of pyrethroid

resistance in *An. funestus* from Likoma Island, on Lake Malawi has also been reported [156]. Overall this suggests that this resistance mechanism has spread to Malawi [53]. It is also feasible however, that the insecticide resistance has been selected de novo in Chikhwawa. This could be the result of insecticide use in agriculture, as seen elsewhere [129, 229] together with the gene flow in malaria vectors [230, 231]. Vector species can potentially be exposed to agricultural insecticides through residual run-off into vector breeding sites. In Malawi the range of insecticides used for agricultural activities has resurged recently with the resultant potential increase in exposure of mosquito populations to a broad range of insecticides. There has been a history of extensive application of insecticides in agriculture in the Chikhwawa area through the Malawi government farmers input subsidy programme (FISP), where insecticide, especially pyrethroids, have been heavily disbursed to the farmers at no cost or subsidized rate since 2005 as reported by the Shire Valley ADD personnel (Personal communication). Recently, selection pressure from agricultural use has been implicated in the development of resistance in *An. gambiae* in Burkina Faso [129].

More recently the utilization of insecticides for vector control may have contributed to either de novo resistance or an increased selection pressure for spread of insecticide resistance. The NMCP LLIN distribution programme started in 2007 delivering LLINs to pregnant women during ANC visits and to women who attended EPI clinics with their children. This

has resulted in a household coverage of LLINs of nearly 60% in Chikhwawa (See figure 5.9) [41].

The selection of insecticide resistance has great potential to compromise any insecticide-based malaria vector control programme [232, 233]. The resistance reported here in *An. funestus* and *An. gambiae*, Malawi's major malaria vectors, is of major concern, as the current vector control policy is insecticide based, and uses pyrethroids for both IRS and ITNs.

With the advent of more sophisticated biochemical and molecular assays for resistance detection, it is now feasible to accurately analyse large numbers of insects for a range of insecticide resistance genes and monitor their changes over time [189, 234, 235]. Biochemical mechanisms; AChE, GST, general esterase activity and monooxygenase (p450) were not carried out in this study due to an inadequate cold chain to get the samples to a suitable laboratory. However, the use of novel techniques, including microarray, allow the detection of resistance mechanisms without the need for a cold chain [236, 237].

With the recent real-life example of how the same resistance mechanism observed here in *An. funestus* can be associated with the failure of a control programme in South Africa, the use of pyrethroid insecticides for IRS at this stage in Malawi is not evidence-based, and should be discouraged. The South African example of resistance in *An. funestus* with this mechanism is the only documented vector control programme failure due to insecticide resistance. In South Africa *An. funestus* has successfully been controlled by

IRS with DDT for over 50 years [151]. From the bioassay results, carbamates and organophosphates seem to be more effective at both *An. gambiae* and *An. funestus* than the pyrethroids. It is therefore important to have fuller understanding of all the resistance mechanisms in all vectors and based on that a good insecticide choice and resistance management strategy should be implemented.

6.1.3. **Insecticide Quantification and Quality Assurance**

A successful insecticide based vector control programme is enhanced by strong quality control procedures. Currently the recommended way for monitoring residual efficacy of an insecticide on a surface, post IRS, is via the WHO cone assay [238]. This is logistically challenging, as it requires the maintenance of mosquito colonies and transportation of colonies to the field. As such these assays are rarely done in an operational setting. Under the initiative of the Innovative Vector Control Consortium (IVCC) [239], new tools were developed to more easily carry out quality assurance.

The colorimetric results on wall pads show low-level insecticides (<25mg/m^2) in all the three villages. The colorimetric assays rely on the chemical detection of cyanide released by alkaline hydrolysis [240]. This suggests that inadequate insecticide concentration was applied to the surface. WHO currently recommends a concentration >20mg/m2 [238].The insecticide used by the NMCP for this round of IRS was Morkid with the active ingredient of lambda-cyhalothrin. The formulation is well below the concentration recommended by WHO and is likely not to adhere to the surface of house structures found in Chikhwawa. This would account in

part for the low detection of active ingredient. However, this detection of low-level active ingredient may also have been compounded by poor quality of the IRS round and the length of time (12months) that the wall pads were stored at -20^{0}C before analysis. According to Paine el al (unpublished) colorimetric analysis of the wall pads is ideally to be done one month post spraying.

The major strength of this wall pad work, however, is that it is technically feasible to monitor quickly the quality of individual spray operators and the residual efficacy of the insecticide on a surface. The IQK are a novel tool for monitoring and evaluation of insecticides that have been sprayed on a surface. As the kits are developed for the commercial market they will be looking to record, no spray, under sprayed, correct spray and over spray.

There are a number of confounding factors in this study that will have influenced the outcome not least those discussed. Quality of the actual IRS operator needs consideration. Actual IRS application was not supervised so we have no knowledge if all pads were sprayed or not.

6.2. **Impact of IRS and Study Limitations**

Based on this entomological assessment, it is not possible to show whether the Chikhwawa IRS was successful or had a direct impact on both mosquito abundance and reduction in malaria burden. Contrary to a similar assessment work in Bioko, Equatorial Guinea [46, 160] where continuous entomological monitoring of the IRS showed successful control of all three vectors that were responsible for malaria transmission before the start of the intervention, the Malawi scenario had a lot of logistical and

programmatic limitations rendering our assessment quite unreliable. The collection work started in October 2010 when IRS was planned to commence, however it was delayed until the following year in February/March. This is the peak period for both the malaria vectors in Chikhwawa and the IRS therefore coincided with the natural period of mosquito decline toward the end of rainy season in March/April. This natural fall coinciding with IRS implementation made it difficult to attribute the decline as a direct impact of IRS.

In our continuous monitoring work, we did not have 2009/10 comparative baseline mosquito abundance data. This should have assisted in comparing trends in mosquito abundance in the wake of the delayed IRS. The only comparison therefore was to compare the periods between the years 2011 and 2012. Unfortunately, IRS was not implemented in the year 2011/12 which meant we had no data to compare with, as such our comparisons were only for the 5 month dry period pre-IRS (October 2010 to February 2011) and 5 months post IRS (March to July 2011).

This section highlights and discusses the limitations of this study. Where possible, justifications have been made on the steps taken and suggestions on how the standard procedures should have been improved.

The study lacked a contemporous control village. Ideally a control village, or villages, with similar ecology and disease burden would have been ideal for this study. This would have made good comparison on the numbers of mosquitoes collected and the impact of IRS on local malaria risk pre and

post intervention. However, this was not feasible as NMCP aimed for universal coverage in the district and region with IRS. An alternative control well outside the Shire valley region would have had a different ecology, vector species distribution and disease pattern. It was felt that this would offer no comparison and just add logistical and cost implications.

The selection criteria of households within the selected sentinel sites for species abundance could have been improved. There was need for more stringent setting of pre-selection criteria. Household selection was based on firstly, how permanent it would be before being demolished and rebuilt, as is the case with many temporally dwellings within the area. Secondly, a choice was set for household that had a separate kitchen from the bedroom or main house so that kitchen smoke should not darken the white window trap nor confound with mosquito abundance and migration within the house. This decision was based on anecdotal evidence that suggested that the smoke created by burning biomass fuels inside houses may repel host-seeking mosquitoes [241], although an in-depth literature review found little evidence that smoke from fires led to a corresponding reduction in malaria [223]. This criterion created a bias in the selection and it would have been better if we had randomly picked any house within the quadrant of interest regardless of any other physical factors. Finally, household selection was based on consideration of houses with minimal open eaves to allow for easy mounting of the exit window trap. Usually houses with large open eaves are temporary and easily abandoned and

therefore not ideal for the collection process. While the selection of closed eaves may be seen as a limitation, because these houses may not have been representative over the overall housing structure in the village, this did help the accurate collection of mosquito abundance trend over time as there would be less variation attributed to the entry and exit through the eaves.

The third limitation to the study was that we only made a single assessment of IRS spray round pre and post spray. Ideally it would have been worthwhile to cover at least two consecutive spray rounds to ascertain whether the mosquito abundance trend is really as a result of the IRS intervention or other confounding factors. This was not possible because the second round of IRS was delayed. Initially, the second IRS round was planned to be implemented from October 2011 but it never happened until the following season 2012/2013. This therefore meant we had a single data set to compare with i.e. pre and post IRS of 2010/2012. However, because the study covered two rainy seasons of 2010/11 and that of 2011/12, we managed to make a comparison of the mosquito abundances between these two consecutive seasons.

Timing of the spray round was another limitation. Ideally IRS is supposed to be conducted at the onset of the rainy season i.e. October for Chikhwawa, however the first IRS was delayed to the following year until February right within the rainy season. The delayed IRS may have affected the overall community level IRS coverage negatively, as there were reports of households declining participation because it required them to put their

furniture outside in the rain. Similarly, this would also compromise the natural trends of mosquito abundance, as it occurred at time of declining abundance, and it is impossible to attribute the decline to the IRS for this reason.

The quality assurance of data collection conducted independently by household owner may raise concerns of bias in this study. However, we attempted to address this by thorough training on mosquito collection to every household owner undertaking the daily mosquito collections. Safety information on handling of isopropanol, and other collection material as well as practical illustration of the entire mosquito collection and recoding process was performed at every household and the household owners demonstrated independence in doing the entire process. Routine refresher trainings and general update meetings were facilitated at every monthly visit to enhance quality of the collection and identify and resolve potential problems encountered by the household owners in the course of the collection. Data from *Culex* species or any flies collected in the window exit traps assisted in confirming that collection was indeed taking place. Furthermore, routine and surprise spot check visits were conducted for quality control purposes to check whether the household owner was collecting the mosquitoes and documenting the collections as per study operating procedures.

On WHO bioassays performed in the study, a notable limitation was low numbers of mosquitoes that were sometimes subjected for the experiment. Ideally, controlled triplicates of at least 25 mosquitoes per

bottle are recommended for the bioassays [219]. However depending on the seasonality and availability of the adult indoor resting mosquitoes, as was the case, it was difficult to meet the prescribed numbers of the F1 generation at goal. This then meant that sometimes we were either working with fewer mosquitoes or sometimes completely redoing experiments at a later time. Our findings were however consistent with those from other groups assessing insecticide resistance in the same area [52] signifying the reproducibility as well as the evidence of pyrethroid resistance in the *Anopheles* spp.

As regards comparable clinical data for the study, a notable limitation was that parastiaemia data was collected from 50 villages in the area, and the data from the 3 villages involved in the entomological study was low. A direct comparison between parasitaemia and entomology in the 3 villages was not possible, due to low sampling points from the MIS survey [213] However, the likely trend in parasitaemia presented, likely reflects the entomological findings.

Overall, despite these limitations, the conducted study adds valuable information on the changes in abundance, insecticide resistance profile and insecticide quantification as an easy way to measure surface concentrations of insecticide to ensure good quality spraying. The study results on resistance is consistent with findings from other studies within the same region [52, 157, 211] and neighbouring countries and describe a low-cost approach that adds considerable information in a setting where a randomized controlled design was no longer an option.

The impact of IRS on mosquito abundance and malaria burden is not clear as partly suggested by the pre-post data comparison because of among others the limitations described above. However, the low numbers of mosquitoes in the succeeding spraying season (albeit not implemented) suggests that 2010/11 IRS might have crashed the mosquito population and that the breeding numbers for the succeeding seasons were still lower to create an upsurge in the numbers

6.3. **Conclusion**

In this high transmission area, continuous entomological surveillance proved low cost complementary monitoring and evaluation tool to assess the impact of IRS. Essential to the success of the IRS campaign is the implementation of strong quality control procedures that facilitate the assessment of programmatic effectiveness in a simple and manner. It is therefore important to incorporate entomological and burden monitoring and evaluation assessments to accurately monitor short term impact on vector control efforts.

7. APPENDIX 1

WRITTEN INFORMED CONSENT FORM (English)

Vector Assessment

Informed Consent Form for: Household owner

Title of Project
ACTia - Vector Population Monitoring Tool (VPMT)

Part 1: Consent Explanation
[To be read and questions answered in the volunteer's local language].

Participation Information:
This project is part of ACTia (safety and effectiveness of combination therapies with repeated treatments for uncomplicated *P. falciparum* malaria) currently operating in this area. This entomological component is being carried out to better correlate the distribution and species abundance of malaria mosquitoes toward safety and effectiveness of combination therapies with repeated treatments and bring a better service to the community as a whole.

We would like to seek permission to have your house fitted with a window trap to collect mosquitoes.

It is very important that you understand the following general principles that apply to all participants in this project:

1) Permission to enter your house is entirely voluntary.
2) Persons may withdraw from participation in this study at any time.
3) Refusal of permission to collect mosquitoes from your house will involve no penalty

[After you read the explanation, please feel free to ask any question that will help you to understand more clearly the nature of the study].

Type of Information/ Data.

1. Mosquito abundance.
2. Types of mosquitoes found in the community.
3. The number of mosquitoes carrying malaria infections

House selection
Six houses in the village will be selected and have window traps fitted.

The house owner will be asked to remove the mosquitoes from the trap every day and empty them into specially prepared tubes.

Procedures to be followed
No humans will be involved in mosquito collection procedures. Once permission is given by the owner of the selected house, a window trap will be fitted to one of the windows by the project team for routine mosquito collection.
[The window trap will not interfere with everyday household duties]

Every day the house owner will inspect the trap, remove mosquitoes with an aspirator and store them in ready prepared tubes for later analysis.

At the end of the month the project team will collect the filled tubes for analysis.

Duration of the study
This activity may take up to 3 years but time frame is dependent on the ACTia's major work plan.

Benefits

To compensate for the time loss in collecting mosquitoes, the house owner will be compensated $10 (or its equivalent in Malawi Kwacha) per month.

Assurance of confidentiality:

No information on private life will be recorded or discussed with anyone. You will receive a copy of this consent form.

Questions
[If there is any section of this consent explanation sheet that you do not understand, you are welcome to ask the investigators before signing for explanation].
Signatures

Name of Householder:
|_____|

Signature or: |_____|

Thumbprint (*if cannot write*):

Date:|_____/_____/_____|

Part 2: Certificate of Consent

I, _____, having understood the explanation of the project given do give permission to fit my house with a window trap to collect mosquitoes for the project. The nature, duration, purpose and methods by which the project will be conducted; and the inconveniences and hazards which may be expected have been explained to me in full by _____, and are set forth in the Informed Consent Explanation, which I have signed (and received a copy). I have been given an opportunity to ask questions concerning this investigational project, and any such questions have been answered to my full and complete satisfaction. Should any further questions arise, I may contact **Dr Michael Coleman or Benjamin Nyoni at 099 5825 417**.

I understand that I may at any time during the course of this study refuse permission to collect mosquitoes from my house prejudice.

Name of Householder: |_____|
Signature: |_____|

Thumbprint (if cannot write):

Date: |_____/_____/_____

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